Ethology and sensory physiology associated with social organisation in yellow-eyed mullet (*Aldrichetta forsteri*), kahawai (*Arripis trutta*), and snapper (*Chrysophrys auratus)*

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by

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I certify that no material has previously been submitted and approved for the award of a degree by this or any other University.

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List of abbreviations

2D	Two-dimensional
3D	Three-dimensional
BL	Body length
CN	Canal neuromast
LLS	Lateral line system
NND	Nearest neighbour distance
SA	Separation angle
SEM	Scanning electron microscopy
SN	Superficial neuromast

1 General Introduction

1.1 Theoretical framework for collective behaviour

Collective behaviour is common throughout the animal kingdom. Many of us will have witnessed the synchronised efforts of flocks of birds, swarms of bees or schools of fish, and marvelled at this wondrous phenomenon. Of all animals in the animal kingdom, no other group displays a greater range of collective behaviour than fish (Vicsek & Zafeiris, 2012). Presently, there are around 30,000 catalogued species of fish, and they represent the highest diversity of all vertebrates (Eschmeyer et al., 2010). Interest in *why* and *how* fish aggregate can be traced back to the early 1900s with seminal work by Parr (1927), which became the theoretical foundation of understanding this behaviour. Since then, a great deal of research has amassed to describe the types, structure, function, maintenance, and evolution of collective behaviour in fish groupings (Bell, 2013; Herbert-Read, 2016; Katz et al., 2011; Partridge, 1982; Pitcher, 1986; Williams, 1964). An example of collective behaviour in fish is shown in Figure 1.

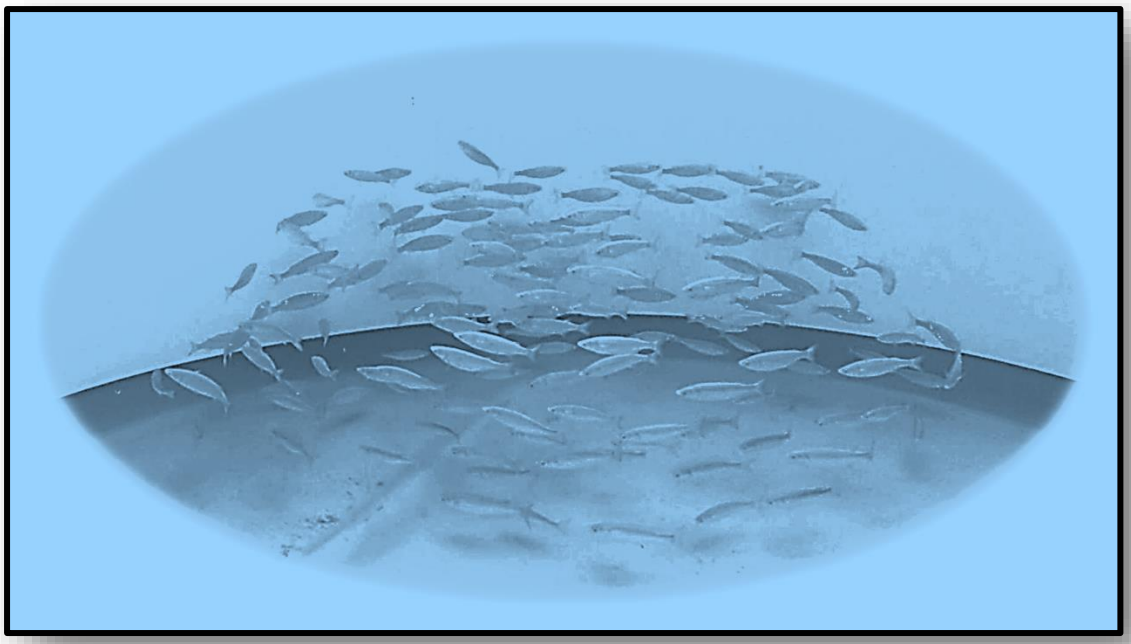

Figure 1 Collective milling behaviour of yellow-eyed mullet (*Aldrichetta forsteri*) as a predator avoidance response. Photo credit KL Middlemiss.

Thirty years after the pioneering work of Parr (1927), Breder (1959) consolidated the knowledge about social living in fish. From this, he derived 27 key points and highlighted that collective behaviour involves a high level of complexity relating to both species and environment, and that only the surface of this complexity had been scratched. This finding has since been reinforced by continued global research effort over the following decades. This started to change in the late 1900s, with studies beginning to investigate interactions rules associated with the complexities of group living (Parrish & Turchin, 1997; Partridge, 1982; Pitcher, 1986; Shaw, 1978), the mechanisms of which have now been succinctly reviewed (Krause & Ruxton, 2002; Pavlov & Kasumyan, 2000), and include a defined set of rules closely linked to sensory systems, and information transfer. More recently, research has focussed on the role of structural dynamics involved in group interactions (Katz et al., 2011), inter-individual interaction rules (Tien et al., 2004), social learning (Brown & Laland, 2006), effects

of group size and phenotypic assortment (Krause et al., 2000; Rieucau et al., 2014a), and the role of sensory modalities (Montgomery & Carton, 2008). Despite significant progress in these areas, there is still much more known about the *why* than about the *how*, and the following elucidates details of each.

In addressing the question of *why* fish display collective behaviour, we first consider the evolutionary underpinnings. Collective behaviour evolved as a result of two main selection pressures, *foraging* and *predation* (Partridge, 1982), and therefore, is an evolutionary survival strategy essential to individual fitness (Ballerini et al., 2008). The reality is that around 50% of fish display this behaviour at some stage of their life-cycle (e.g. juveniles or adults) (Krause & Ruxton, 2002; Shaw, 1978), and it is particularly prevalent in migratory species such as sardine schools (Coetzee, 2000). The sheer number of species displaying this behaviour suggests a considerable advantage to individual fitness. *Foraging* success rates increase with group size; the many eyes hypothesis suggests that the more individuals looking, the quicker the group will find unevenly distributed food resources (Pitcher et al., 1982; Powell, 1974; Ranta & Juvonen, 1993). Therefore, the more neighbours you swim with, the more likely you are to find dinner. Reduced *predation* risk via group living is all about safety in numbers (Lima, 1995). A key mechanism supporting this theory is the 'oddity' effect where *predation* response is delayed or confused (Landeau & Terborgh, 1986; Radakov, 1973; Shaw, 1978; Tien et al., 2004). Having many individuals to choose from introduces a confounding element into predator-prey interactions, resulting in predators having less foraging success. Essentially, swimming with lots of other fish reduces the likelihood you will become dinner. In addition to *foraging* and *anti-predator* benefits, group living may also confer advantages via reduced energy use through improved *swimming efficiencies* (hydrodynamics) gained through slipstreaming (Hemelrijk et al., 2015; Weihs, 1973), and navigation (Simons, 2004). These benefits to group living balance any negative consequences, such as competition for food

resources among group members. Anti-predator behaviours displayed by the group involve coordinated evasive tactics [e.g. fountain effect where the group splits in two and regroups behind the predator (Hall et al., 1986; Magurran & Pitcher, 1987; Marras et al., 2011; Rieucau et al., 2015a)]. However, the ability to perform coordinated swimming behaviours is specific to a particular grouping of fish.

Prior to the 1980s, all fish groups were heuristically described interchangeably as *schools* or *shoals* (Spooner, 1931) (Fig. 2). However, these terms describe two distinctly different functional groups, as was recently reviewed by Delcourt and Poncin (2012). Contention over the categorisation of fish groups can be traced back as far as Parr (1927), and it was proposed by Shaw (1978) that schools only exist as mutually attracted individuals. But important clarification by Pitcher (1983) resulted in the now commonly accepted definitions that *schools* are synchronised (uni-directional) and polarised (aligned), whereas *shoals* display mostly unsynchronised, but social behaviour. Therefore, by definition, synchronised group behaviour results from the formation of '*schools'* not '*shoals'*. So *how* is it possible to achieve the great feats of uniformity seen in *schools* of fish, which serve as such a vital evolutionary adaptation in the survival of so many teleost species? What are the mechanisms which underlie this behaviour?

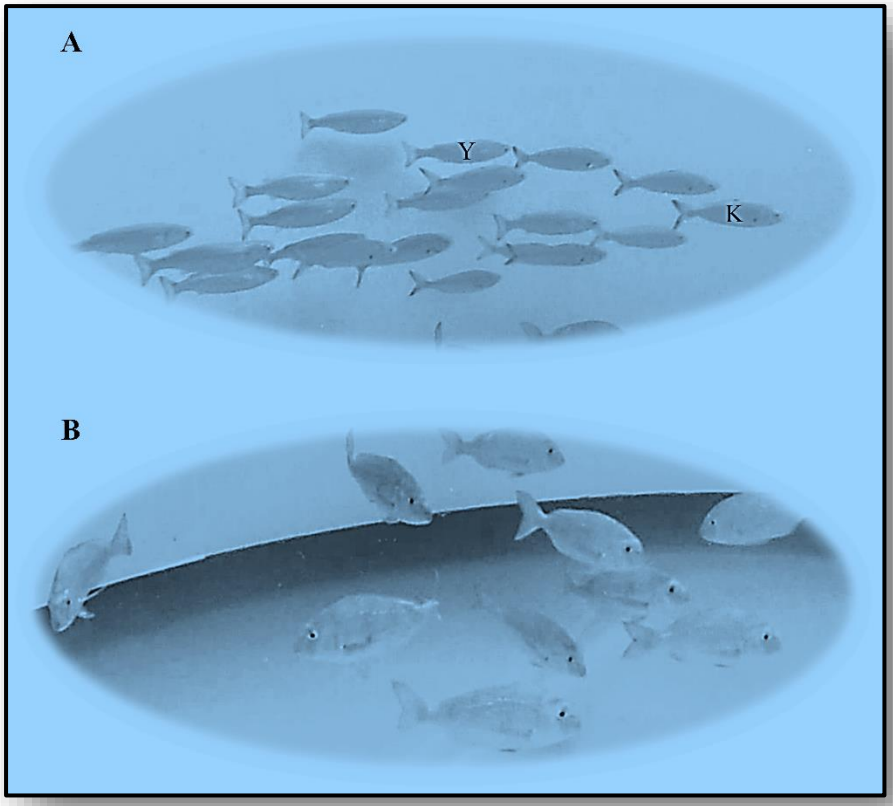

Figure 2 Representations of school (A) and shoal (B) formations. Species shown are yellow-eyed mullet (*Aldrichetta forsteri*) and kahawai (*Arripis trutta*) (A), and snapper (*Chrysophrys auratus*) (B). Abbreviations: Y, yellow-eyed mullet; K, kahawai. Photo credit KL Middlemiss.

Contrary to initial views (Radakov, 1973), fish schools are dynamic structures. Belying their seemingly effortless unified appearance is a constant state of flux, and a very deliberate state of self-organisation (Breder, 1954). Coming in numerous shapes and sizes (e.g. oblong and ellipsoid), groups range in size from ten to millions (the latter usually being pelagic species such as herring), and can span several kilometres (Makris et al., 2006; Misund, 1993; Partridge et al., 1980; Pavlov & Kasumyan, 2000; Pitcher et al., 1996). It is remarkable that thousands of unrelated individuals can achieve unified behaviour in the apparent absence of a leader to provide direction. Particularly in large schools, in order to achieve cohesive behaviour they require consensus and conformity by all individuals based on a defined set of interaction rules (Paramo et al., 2010; Partridge, 1980; Pitcher & Partridge, 1979; Sumpter et al., 2008). These

rules are derived from local (e.g. immediate neighbours) and global properties (e.g. group size) (Rieucau et al., 2015a), the application of which requires a specific set of behavioural traits to facilitate maintenance of group structure. Seminal work by Breder (1954) first elucidated that local interaction rules are governed by attraction and repulsion forces. Simply put, fish move away from each other at close proximity and towards each other when separation distances become too great (Couzin et al., 2002). The end result is that a combination of emergent properties (Parrish et al., 2002; Viscido et al., 2004) constitute the group as a whole; in other words, group behaviour is the sum of many independent behavioural characteristics displayed by each fish, and the interactions among them. Effectively, this constitutes the 'rule book'.

Individual behavioural interactions are primarily guided by three emergent properties: optimal nearest neighbour distance (NND), separation angles (SA), and swimming velocity (Herbert-Read, 2016; Rieucau et al., 2015a; Tien et al., 2004). Briefly explained, NND is the distance from a focal fish to the next fish in closest proximity, SA the degree of polarity among individuals (alignment), and swimming velocity is directional swimming speed (Viscido et al., 2004). With respect to NND, there is a pronounced level of spatial isotropy (uniform distribution of individuals) within groups among species (Herbert-Read, 2016). The rule of thumb is that a focal fish prefers a minimum distance of around 1 body length (BL) to the nearest neighbour (Partridge, 1982), for example, NNDs of 0.9 BL in minnow (*Phoxinus phoxinus*) (Partridge, 1980), and 0.82 BL in herring (*Clupea harengus*). The angle of separation between individuals is similarly uniform among schools, and fish also constantly alter swimming velocity in order to help maintain optimal NND and SA within the school (Partridge, 1982).

Each individual applies these three interaction rules in response to sensory cues (e.g. visual and mechanosensory (Bleckmann, 1986). These are generated by changes in immediate neighbour behaviour when responding to external stimuli (e.g. predator-prey interactions, foraging)

(Herbert-Read, 2016), and information is quickly propagated like a wave through the whole group as shown in anti-predatory behaviours of Atlantic herring (*Clupea harengus*) (Marras et al., 2011). This requires a suite of sensory modalities to detect and facilitate the transfer of information throughout the group. Therefore, the neurobiology underpinning sensory modalities in fish is a key mechanism in maintenance of schooling behaviour. It is suggested that integrating knowledge on behavioural interactions within an evolutionary, physiological and neurological framework is paramount to unravelling the intricacies of collective behaviour (Hofmann et al., 2014).

Fish use an integrated approach to sensing the aquatic environment, drawing on input from various sensory systems (Montgomery & Carton, 2008). For schooling behaviour, this predominantly involves the lateral line system (LLS), and vision. Examples of the use of these systems in group behaviour are found in saithe (*Pollachius virens*), and Mexican blind cave fish (*Astyanax mexicanus*) (Kowalko et al., 2013; Partridge & Pitcher, 1980). The LLS is a mechanosensory system unique to aquatic environments (Montgomery et al., 2014; Webb, 2011) consisting of neuromast cells, located on the skin or in subdermal canals (Blaxter, 1987), which contain hundreds of tiny hair cells used to detect hydrodynamic changes in the surrounding water (Bleckmann, 1986). Visual function is closely associated with the optic lobes (tectum) in the brain (Springer et al., 1977). Asymmetrical brain hemispheres have given rise to side biases, including vision (i.e. preference for left or right eye use) (Bisazza & Brown, 2011), and is termed lateralisation (Rogers et al., 2004). This has benefits for schooling fish.

Fish position themselves within a group to best engage the use of these sensory modalities (Hemelrijk & Hildenbrandt, 2012; Herbert-Read, 2016). In other words, they position themselves where they can best sense (e.g. see and feel) changes in neighbour behaviour (e.g. directional change), which in turn results in self-adjustment. However, these systems are not able to be applied to schooling behaviour at all times, and efficacy is impacted by changes in

abiotic factors, such as photopic conditions (Ryer & Olla, 1998), turbidity (Borner et al., 2015), and water flow (Engelmann et al., 2002). Effectively, conditions in the surrounding water dictate which senses can be utilised, and this directly impacts how schools maintain structure.

There is one set of interaction rules to guide the social relationships between individuals within groups once they have formed (e.g. NND, SA), and another before formation takes place. Typically, the latter includes decision making around phenotypic similarity (e.g. fish size) (Hoare et al., 2000). The advantage in phenotypically-assorted groups is that if you look like your neighbour, you are less likely to stand out to predators; this is known as the oddity effect (Ranta et al., 1994). Therefore, this behavioural trait can improve individual fitness. Even mixed-species groups, for example shoals of golden shiner (*Notemigonus crysoleucas*) and banded killifish (*Fundulus diaphanous*) (Krause et al., 1996a), which arguably, by their nature, do not fit the reduced oddity theory, show levels of size-assortiveness within the shoal. Therefore, predation selection pressures drive phenotypic assortment, because they confer fitness advantages. For assortment to occur, groups must first have the option to stay/leave the group. Therefore, group encounters are key components of group formation (Croft et al., 2003). Small geographical areas, such as estuaries, can lead to increased group encounters (Flierl et al., 1999), and therefore increase the opportunity for phenotypic assortment. Understanding the effects of these encounters, in terms of decision making around group formation, has important implications for elucidating the mechanisms that influence group interactions and anti-predator/foraging behaviours in natural fish populations. The application of this knowledge has potential synergies with global efforts to provide more sustainable fish capture methods.

Global demand for seafood production continues to rise, and has increased by more than 5-fold since the 1950s (FAO, 2016a). Traditional commercial fishery methods have typically involved trawl and purse seine nets (Prosser, 2015; Wardle, 1986). Whilst these methods capture high numbers of fish, they are financially costly (e.g. boat operating costs), not species-specific and

thus potentially unsustainable (Halldorsson et al., 2012; Wilson et al., 2014). This, combined with the increasing global demand for seafood, has fuelled investigations into the development of new methods to sustainably increase the productivity of commercially important fish species. Recently, these methods have included the conditioning and capture of free-ranging fish via anthropogenic feeding stations. These feeding stations have been shown to aggregate and, in some cases, to enhance fish growth (Bjornsson, 2011; Mustafa, 2003; Taylor et al., 2017). This method has also been referred to as sea-ranching or herding (Mustafa, 2003).

The potential for anthropogenic feeding stations has only been explored in a handful of teleost species. For example, an anthropogenic feeding station enhanced recruitment of the Black sea bass (*Centropristis striata*) (Lindell et al., 2012), as well as sustained recruitment and growth in free-ranging Atlantic cod (*Gadus morhua*) (Bjornsson, 2011). Even less is known about the community structure or feeding station behaviours of multi-species aggregates, such as those found in estuarine fish populations. Estuarine habitats provide overlapping resource use (e.g. shared food sources) to many commercially and recreationally important New Zealand fish species (Morrison et al., 2014), including the current study species; yellow-eyed mullet, snapper and kahawai. Therefore, supplementary feeding, via estuarine-based anthropogenic feeding stations, has the potential to attract multiple foraging species. Shared resource use in estuarine habitat may also increase inter-species competitive interactions and/or attract predators, both of which could negatively impact the success of feeding stations. Understanding how interspecific behaviours (e.g. foraging hierarchies and group structure) may modulate the effect of feeding stations currently remains largely unknown.

1.2 Species ecology and study site

1.2.1 Yellow-eyed mullet (Aldrichetta forsteri)

The yellow eyed mullet (*Aldrichetta forsteri*) belongs to the family Mugilidae, a globally abundant fish family consisting of 71 species in 20 genera, of which yellow-eyed mullet is the only species in the genus *Aldrichetta* (Gonzalez-Castro & Ghasemzadeh, 2016). Remarkably, all Mugilidae species possess very uniform phenotypes (fusiform body shape), and typically grow to a maximum length of around 25–30 cm (Gonzalez-Castro & Ghasemzadeh, 2016). Two mullet species are found in New Zealand; yellow-eyed mullet (Fig. 3), and grey mullet (*Mugil cephalus*) (Hector, 1897). The geographical range of the yellow-eyed mullet includes temperate and tropical waters of New Zealand and Australia (Curtis & Shima, 2005; Froese & Pauly, 2017; Morrison et al., 2014; Paulin & Paul, 2006). The species has a relatively short life-cycle with a maximum age of around seven years, which includes a pelagic spawning phase, followed by migration to shallow estuarine and coastal environments where they are ubiquitous, and considered the most abundant species (Taylor & Paul, 1998). The Mugilidae family are obligate (compelled) schoolers that are often found in large groups (Crosetti & Blaber, 2016). They occupy dynamic habitat (e.g. constant fluxes in tidal flow, salinity, and temperature (Potter et al., 2010)), with overlapping trophic layers resulting in increased predator-prey interactions, and competition for resources (e.g. food) (Whitfield, 2015). In order to exploit these environments, yellow-eyed mullet possess high physiological tolerances for stresses, such as daily changes in salinity (Whitfield & Elliott, 2002), which likely contributes to their abundance. Yellow-eyed mullet are omnivorous and juvenile and adult fish forage mainly on benthic detritus, crustaceans, small fish, polychaete worms, and vegetation such as algae (Taylor & Paul, 1998). This species is considered an estuarine opportunist (Potter et al., 2015), as they fulfil an important ecological role in nutrient cycling of detritus in sedimentary estuarine habitats (Whitfield & Elliott, 2002). Their diet makes them rich in omega-3 fatty

acids (primarily from algae) (Vlieg & Body, 1988), therefore, they provide a valuable dietary source of fatty-acids for predators. Mugilidae commonly reside at the bottom of the food chain, sitting just above primary producers (Vinagre et al., 2012), and yellow-eyed mullet are a prey species of piscivorous teleosts (e.g. kahawai (Baker, 1971)), as well as inshore avian species, such as pied shag (*Phalacrocorax varius*) (personal observation, KL Middlemiss). Due to their abundance and high nutrient value (e.g. high fatty-acid content) to both avian and aquatic predators, they could be considered a key-stone estuarine species. Interestingly, from an economic perspective, given they are high in omega-3 fatty acids, and are found in abundance, it is surprising that they currently form only a very small part of the New Zealand commercial fishery (<50 t annually (Morrison et al., 2014)).

Despite being the most abundant inshore species, little is known about the biology of yellow-eyed mullet. The little that is known comes from studies on feeding behaviour (Coubrough, 2004), growth (Curtis & Shima, 2005; Harris, 1968; Jones et al., 1996), ontogenetic behavioural development (Kingsford & Tricklebank, 1991), estuarine fauna composition (Potter & Hyndes, 1994), lepidology (Liu & Shen, 1991), canal based LLS (Liu & Shen, 1993), sustainable fishing (Thomson, 1957), and lipid content (Vlieg & Body, 1988).

Figure 3 Image of yellow-eyed mullet (*Aldrichetta forsteri*). Photo credit KL Middlemiss.

1.2.2 Snapper (Chrysophrys auratus)

Snapper (Fig. 4), a facultative schooling, demersal fish, also commonly known as sea bream or porgie, belong to the Sparidae family, which consists of 38 known genera (Froese & Pauly,

2017). Previous taxonomic nomenclature for this species was synonymous with *Pagrus auratus* (Gomon, 2008); however, the genus *Chrysophrys* is slowly being adopted by researchers (Parsons et al., 2014), and is the genus referred to in the current study. Snapper geographical range covers the warm temperate waters of the Indo-Pacific encompassing New Zealand and Australia (Froese & Pauly, 2017; Paulin, 1990). In New Zealand, the southern distribution of snapper is limited by water temperature, preferring more temperate Northern waters, and they are found no further south than latitude 43°S (Crossland, 1981). Preferred adult habitats are rocky coastal areas (Parsons et al., 2014), and estuaries for juvenile life stages (Hartill et al., 2003). As adults, they seasonally migrate between inshore and estuarine habitat, but show high fidelity rates to inshore habitat (Crossland, 1981). Juveniles utilise estuarine nursery habitats for refuge and rich food resources (Hartill et al., 2003), which creates overlapping and competing resource use with other estuarine inhabitants, such as yellow-eyed mullet. They are a commercially important New Zealand species constituting the fifth largest inshore fishery, worth $ND265 million annually to the fishing sector, and form the largest recreational fishery (Statistics New Zealand, 2008). Snapper are long-lived (>60 years) (Francis, 2012), and are a predatory species with a benthic and pelagic diet consisting predominantly of crustaceans, shellfish, and fish (Colman, 1972; Godfriaux, 1969; Morrison et al., 2014). Being an estuarine associated species their diet also includes yellow-eyed mullet (personal correspondence, D. Cook). Snapper are broadcast spawners (Morrison et al., 2014), and many (but not all) schools migrate to spawning grounds during the warmer summer months (November–March in New Zealand) (Parsons et al., 2014).

Figure 4 Image of snapper (*Chrysophrys auratus*). Photo credit KL Middlemiss.

1.2.3 Kahawai (Arripis trutta)

Kahawai (Fig. 5) are a member of the Arripidae family, of which *A. trutta* is one of four species in the genus *Arripis*. *Arripis* is the only genus within the Arripidae family. They are endemic to New Zealand, with a geographical range extending from the far North (29° S) to the far South (46° S), including the Kermadec and Chatham Islands (Crossland, 1981). Similar in fusiform body shape to yellow-eyed mullet, kahawai are a pelagic species and display size-assorted schooling behaviour, forming groups ranging from hundreds to several thousand individuals (Morrison et al., 2014). Interestingly, there are reports of kahawai forming mixed schools with mackerel in the Nelson region (Jones et al., 1992). The maximum reported age is around 26 years, growing to lengths of up to 89 cm. Their life-cycle includes a pelagic larval stage (kahawai are unconfirmed serial pelagic batch spawners), before utilising estuarine nursery habitat as juveniles, and migrating between coastal/estuarine in adult stages (Morrison et al., 2014). Kahawai are predatory piscivores with a diet predominantly including crustaceans, and several pelagic fish species, including yellow-eyed mullet (Baker, 1971; Morrison et al., 2014).

Figure 5 Image of kahawai (*Arripis trutta*). Photo credit google images.

1.2.4 Study site

For the duration of my doctoral studies, September 2014 to September 2017, I was based at the Plant & Food Research Seafood Research Facility, Port Nelson, New Zealand (Fig. 6). Wild fish collected for use in the current study were caught from the surrounding Nelson Haven estuary (Fig 6B). The estuary is around 1300 ha in area, fed by the Maitai River, and bounded by coastline and a naturally forming 13.5 km long boulder bank (Gillespie, 2008). A shallow estuary, the Nelson Haven, in the upper reaches, drains completely on ebb tides, and has a tidal range between <1.2 m (low) and >4.2 m (high) (Gillespie, 2008). This generates a range of dynamic abiotic conditions, such as daily/hourly fluxes in temperature and salinity (Potter et al., 2010). Therefore, aquatic species must possess a range of physiological, sensory and behavioural abilities in order to successfully exploit these challenging environments. Intertidal estuarine sand flats support species of aquatic vegetation important to teleost species, including eelgrass (*Zostera* sp.), glasswort (*Sarcocornia quinqueflora*), and algae (Gillespie, 2008). The estuary is an important nursery habitat for several fish species (including the three study species), providing food resources and refuge. It is of significant cultural, historical, geological, ecological and recreational significance to the Nelson region (Gillespie, 2008). From an entire ecosystem services perspective, it provides rich food sources for all trophic levels of the food web, including avian (e.g. pied shag) and fish species (e.g. yellow-eyed mullet, snapper and kahawai). Species richness of fish inhabiting the Nelson Haven estuary creates overlapping,

and often competing resource use, which results in both inter and intraspecific interactions between both avian and aquatic species.

Figure 6 (A) topographical map of Nelson, New Zealand with the Nelson Haven estuary highlighted by solid red line, and (B) The New Zealand Institute for Plant & Food Research Limited Seafood Research Facility, marked with red circle. Photo credit Solander (B).

1.3 Research objectives

The motives for collective behaviour in fish are well known and previously discussed, but predominantly include fitness benefits associated with decreased predation risk and increased foraging success. However, the mechanisms underpinning the behavioural interactions

between individuals engaging in collective behaviour are less well understood. There is a current lack of knowledge to explain the complexity in feeding and predatory relationships in consociated fish species in New Zealand estuarine habitat ecosystems. Specifically, no published research exists on the effects of group size, phenotypic traits or sensory abilities on the rules pertaining to inter- and intraspecific interactions in three commonly associated New Zealand estuarine species; namely the yellow-eyed mullet, snapper and kahawai. Understanding the foraging and predation selection pressures driving group formation behaviour will help elucidate how decision-making rules impact individual fitness in these dynamic ecosystems.

Chapters 3–8 of this thesis present detailed data, under conditions of foraging and predation, on the vision and mechanosensory capabilities of yellow-eyed mullet, the effect of group size, group encounters and phenotypic assortment on structure in groups of yellow-eyed mullet and snapper, and the effects of mixed species assemblages on group structure in yellow-eyed mullet, snapper and kahawai. These research chapters conclude with field observations in chapter eight drawing on all the information learned from the previous chapters, to compare behaviours seen in naturally occurring estuarine populations at an anthropogenic feeding station. The techniques used to collect and analyse data include the use of highly accurate two-dimensional (sonar) and three-dimensional (3D) (stereo-video) measurement systems, and associated measurement software. Accurate estimation has been a technological limitation in early studies on collective behaviour. In combination, the six research chapters aim to answer the following questions:

1. What sensory, eco-physiological and behavioural factors contribute to group formation and interactions in consociated species?
2. How do foraging and anti-predator behavioural traits associated with species affect individual fitness at an anthropogenic feeding station?

To address these questions, the six research chapters utilised both controlled and field–based experiments/observations. Chapter three begins with investigation of mixed-species assemblages to identify behavioural interactions in sympatric species yellow-eyed mullet, snapper and kahawai. This was done to elucidate the complexities of interactive feeding and predatory behaviours in sympatric populations. Chapter four examines the effect of increasing group size on school structure in yellow-eyed mullet to investigate changes in isotropic spatial patterns related to group size. Results will highlight the importance of local and global properties as drivers in maintenance of group structure in schooling fish. Chapter five further describes factors influencing group structure by investigating the effects of phenotypic assortment as a result of group interactions in yellow-eyed mullet and snapper. This information has important implications for understanding the underlying mechanisms influencing foraging and anti-predator behaviours in natural fish populations. Chapter six is the first of two research chapters investigating sensory modalities involved in collective behaviours, in order to describe possible mechanisms for these behaviours. This chapter examines the lateralisation of visual function in yellow-eyed mullet, through anatomical investigation of optic lobe (tectum) morphology, and then relates findings to the role of vision in schooling behaviour. Results from this chapter will elucidate the ontogenetic plasticity of visual functional behaviour–related maintenance of school structure. Chapter seven further investigates sensory mechanisms in maintenance of yellow-eyed mullet group behaviour. Morphological examination describes the structure and function of the lateral line system, and identifies the effects on cohesive schooling behaviour during ablation of this important mechanosensory system. Findings from this chapter will greatly improve our understanding of the role this sensory system plays in collective behaviours. Finally, chapter eight provides field based evidence of the behavioural interactions in naturally occurring sympatric estuarine populations associated with foraging activity at an anthropogenic feeding station operating in

the Nelson Haven estuary. This will allow the knowledge learned from the previous data chapters (3–7) to be extrapolated to wild populations, which is a limitation of previous ethological studies of fish. Chapter 9 provides a summary of all results describing how they addressed the research aims and contributes to the overall body of knowledge of collective behaviour in fish species. It then concludes with future directions and management implications.

2 Photogrammetry methodology

2.1. Photogrammetry theoretical background

Photogrammetry is the use of imagery to make measurements (e.g. fish length), and in aquatic environments involves the use of underwater camera and sonar equipment. The very first underwater photography can be traced back to 1856 (Baker, 1997) and another 67 years passed before the development of underwater video in 1923 (Taves, 1996). Sonar, an echo-sounding technique, uses sound waves for underwater object detection and converts the returning echoes into digital images. Aristotle was arguably the first scientist to discover that sound travels underwater; however, it wasn't until the early 20th century that research effort increased considerably on the development of sonar techniques (Theberge, 1989). Modern sonars are comparable to medical ultrasounds and operate at much higher frequencies than previously used for early fisheries acoustics (e.g. 3.0 mHz vs 200 kHz). This means they are highly sensitive to changes in acoustic impedance (physical properties of an object that the sound waves encounter) and provide very clear imagery. Early photogrammetric imaging analyses were restricted to two–dimensions (2D), and indeed sonar imagery is still made in 2D. There have been many recent technological advances in both systems that have improved image quality and, therefore, subsequent accuracy of analysis. The genesis of 3D stereo-video image analysis techniques in the 1990s (Harvey & Shortis, 1995; Shortis et al., 2009) has led to low-cost, reliable and accurate 3D imagery analysis (Letessier et al., 2015). Testing of the accuracy of imagery equipment, both sonar and 3D stereo-video cameras, used for photogrammetric assessment of fish schools in this thesis is detailed in the following sections. The use of the term accuracy, as defined by Zar (1984), is the ratio of the difference between the actual length measurement vs the estimated length measurement.

2.2. Equipment

2.2.1. Stereo-video camera configuration

The camera system used purpose built camera housings designed to physically confine the cameras so their relationship to the housing port was stable (even when housings were opened to access the camera). The camera housings are robustly mounted to maintain the stereo configuration of the system. The system was assembled from two GoPro® (Hero 3+ silver) cameras (1920 x 1080 p resolution, 60 frames per second, and medium field of view) positioned at a lens separation distance of 420 mm, each with an incline of 4° and contained within waterproof housings affixed to a base plate (Fig. 1).

Figure 1 Photo of an underwater stereo-video camera system consisting of two GoPro cameras (Hero 3+ silver) contained within waterproof housings, permanently affixed to a base plate at a lens separation distance of 420mm, and each camera positioned at an incline of 4°. Photo credit: KL Middlemiss.

This permanent camera system was configured to enable stereo-overlap for image matching in both cameras to fit the dimensions of the observation tank used for all fish observations (3.5 m wide, 2.6 m deep, and volume 13,000 L) (Table 1).

Table 1 Approximate area of stereo overlap at increasing distances from stereo-video camera system.

Range from cameras (m)	Horizontal range(m)	Vertical range (m)
1.0	1.0	0.7
1.5	1.6	1.0
2.0	2.3	1.3
2.5	2.8	1.6
3.0	3.3	2.0
3.5	3.8	2.3

Cameras were calibrated by SeaGIS (2017) using a 500 mm x 500 mm x 300 mm calibration cube, and CAL software (Fig. 2), which determined the geometric characteristics for individual cameras and the physical positioning of each camera relative to the other (Shortis et al., 2009). Calibration accuracy was verified using repeat measures of scale bars of known lengths, at varying distances and rotation angles, resulting in a standard deviation of ~1 mm (personal communication KL Middlemiss and SeaGIS).

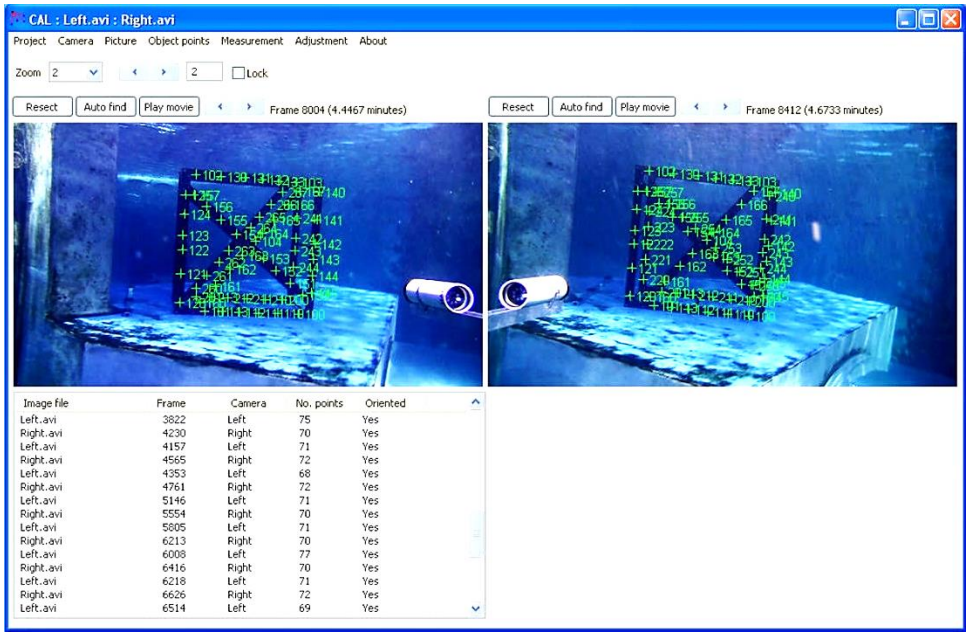

Figure 2 Image of a three-dimensional calibration cube viewed with CAL software (SeaGIS, Australia) used to calibrate an underwater stereo-video camera system. Image credit: SeaGIS, Australia.

2.2.2.1 Stereo-video measurement software

After stereo-video recordings were made, EventMeasure software (SeaGIS, 2017) was used to make 3D photogrammetric measurements directly from video footage (Fig. 3). GoPro video footage was converted from MP3 to Xvid AVI format using Xilisoft® software prior to upload into EventMeasure.

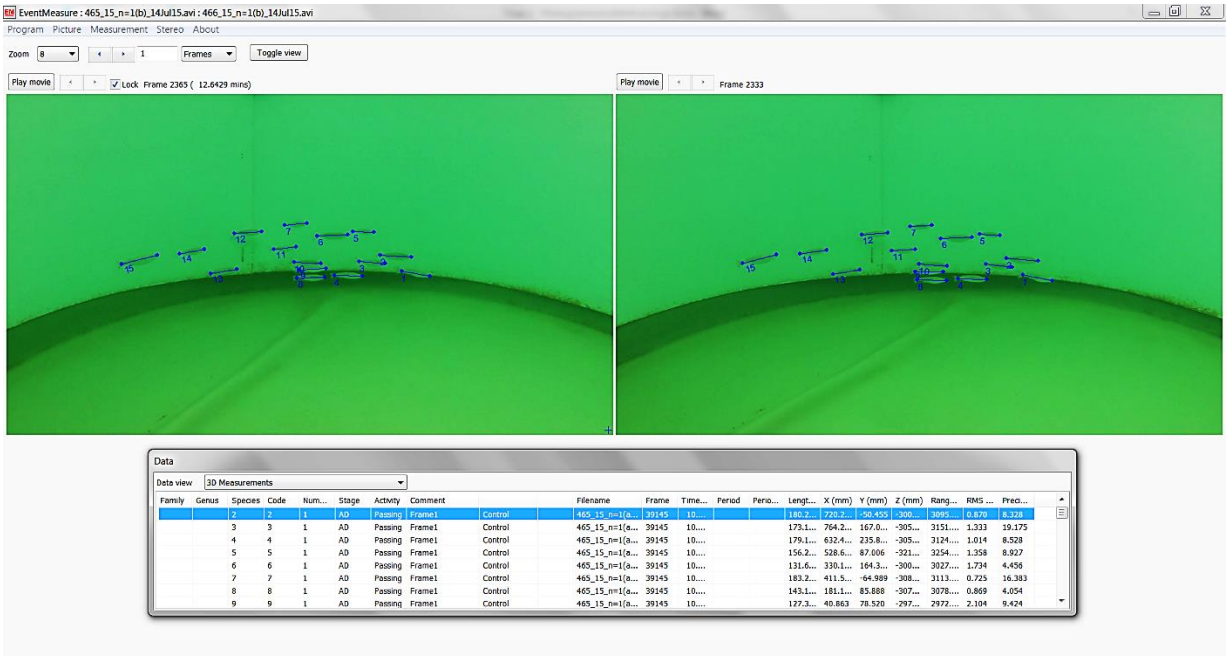

Figure 3 Image of EventMeasure software (SeaGIS, Australia) showing left and right camera images with three-dimensional length measurements of fish represented by blue lines. Image credit: KL Middlemiss.

2.2.2. Sonar configuration

Two-dimensional sonar images were made of both tank-based and wild fish observations using an ARIS Explorer 3000 sonar and ARIScope recording software (Sound Metrics, USA) (Fig. 4). The sonar consists of 128 beams and operates at 3 MHz providing high performance imagery up to 35 m, and is pre-calibrated in accordance with manufacturer (Sound Metrics, USA) standard calibration procedures.

Figure 4 Photo of ARIS Explorer 3000 sonar (Sound Metrics, USA). Photo credit: KL Middlemiss.

2.2.2.1 Sonar measurement software

Sonar footage was analysed with ARISFish software to make 2D geometric fish measurements from images (Fig. 5).

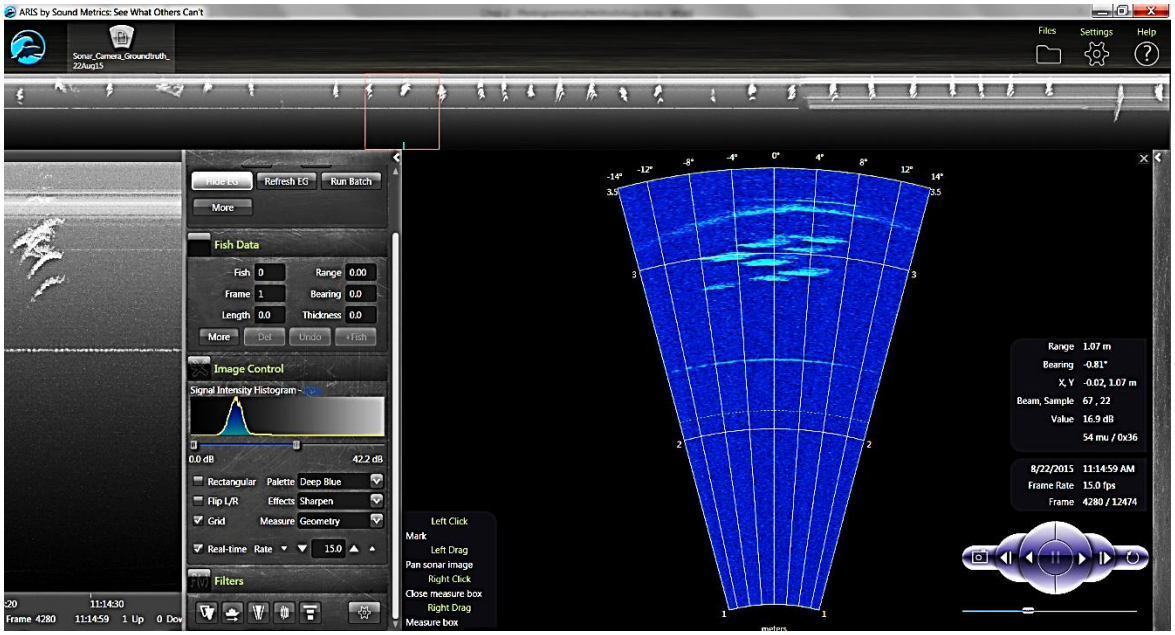

Figure 5 Image of ARISFish software (Sound Metrics, USA) showing an echogram of a fish school. Image credit: KL Middlemiss.

2.2.3. Tank setup

A 13,000 L tank was used (dimensions previously described) for all stereo-video and sonar synthetic target measurements (Fig. 6). To standardise conditions between experiments, seawater was filtered at 1 μm to ensure clarity and visibility to the full tank extent, and water flow was ceased.

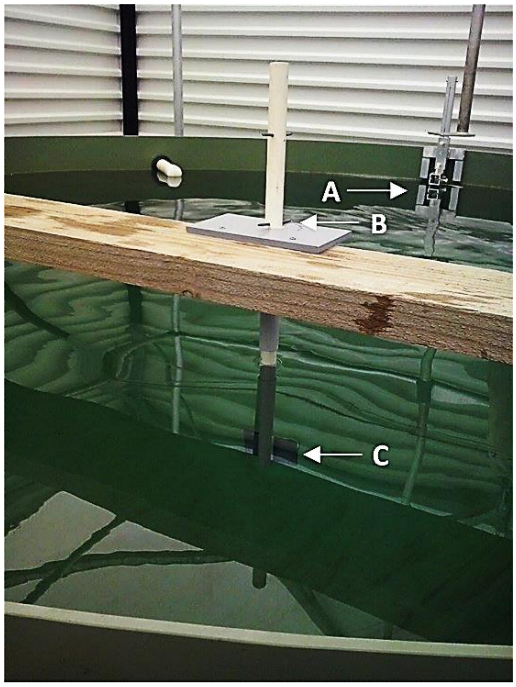

Figure 6 Image showing the setup of synthetic targets for measurement with both stereo-video and sonar imaging equipment. Position of imaging equipment (A), synthetic targets (C) and rotation mechanism (B).

2.3 Sonar and stereo-video calibration verification with synthetic targets

Synthetic targets of three lengths (100, 200 and 300 mm), were positioned at distances of 1.5 m, 2 m, 2.5 m, and 3 m away from the stereo-video camera and sonar imaging equipment and rotated at angles 0°, 15°, 30°, 45°, and 60° clockwise from perpendicular (90°orientation to the camera system). Four repeat measures were made of each synthetic target size, at each distance and rotation angle (except for 60° at 3 m due to physical restriction from tank wall), totalling 59 images and 236 measurements. Measurement accuracy (mean percentage error of the ratio between the known length and the actual measurement) was calculated using the following formula described in Harvey et al. (2002):

$$\text{Error \%} = \sum \left[(M_1 - T_1)/T_1\right] * 100$$

Where M_1 is the estimated length measurement and T_1 is the actual target length.

No statistical analysis was possible between sonar and stereo-video accuracy data due to the differences in measurement characteristics (i.e. 2D sonar vs 3D stereo-video images), therefore, analysis is confined to differences *within* each system. However, comparisons of accuracy are discussed between each system, based on observed data trends. The following details the effects of target rotation angle on measurement accuracy in three synthetic target sizes (100 mm, 200 mm, and 300 mm), at distances of 1.5 m, 2.0 m, 2.5 m, and 3.0 m from the sonar and stereo-video cameras.

2.3.1 Sonar

The most obvious trend seen between the two imaging systems is that sonar image measurements were over-estimates of the actual synthetic target lengths, whereas conversely, stereo-video image measurements were under-estimates (Fig. 7a). Targets presented in a perpendicular aspect (i.e. 90°) to the sonar beams produced the highest level of error measurement. This is likely due to the cross-talk effect between adjacent beams, which is amplified at close range (personal communication KL Middlemiss with Sound Metrics, USA). As target size, distance and rotation angle increased, error rates decreased. Significant decreases in error were found with increased target sizes (100 mm and 200 mm $P < 0.001$, and 300 mm $P = 0.003$, 3-way ANOVA and Tukey test for multiple comparisons). Distance Measurement accuracy of <5% error was obtained from sonar images where targets were at distances of >2.5 m, rotation angles >30°, and target sizes were larger than >100 mm. Findings are consistent with similar tank-based studies (unpublished data) by the Alaska Department of Fish and Game (personal correspondence KL Middlemiss with S. da Costa).

2.3.2 Stereo-video camera

The mean error around estimations of target length using the stereo-video configuration were under-estimations (Fig. 7b). This is similar to findings in Shortis and Harvey (1998). As the rotation angle increased away from perpendicular, error increased; however, there was a

correlation between error rates and target size with significantly less error in target size 300 mm compared with 100 mm and 200 mm (1-way ANOVA Kruskal-Wallis, Tukey test for multiple comparisons, $P < 0.05$). These results clearly showed that the highest level of accuracy was obtained when the target aspect was perpendicular to the camera and therefore the edges were clearly detectable and that in this case, regardless of object size or distance from the camera, an error rate of <3% was achieved. It should be noted that abiotic factors such as photopic conditions and water clarity affect underwater visibility, therefore, error rates can fluctuate as a result (not measured).

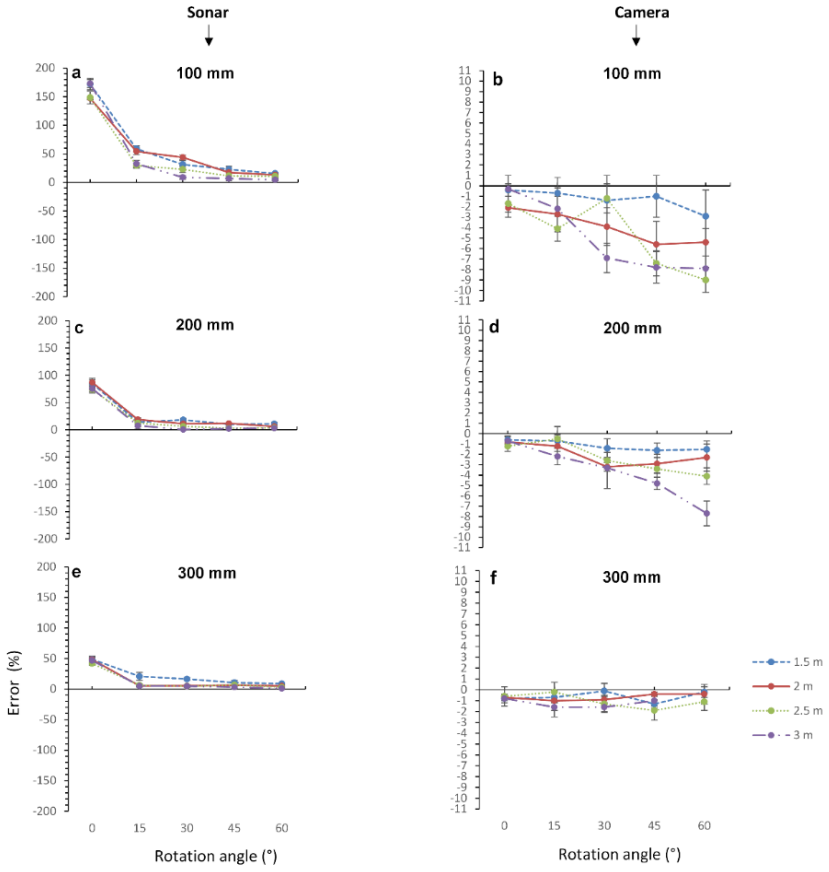

Figure 7 Effect of increasing rotation angle on measurement accuracy for sonar (a, c, and e) and stereo-video (b, d, and f) imaged synthetic target lengths of 100 mm, 200 mm, and 300 mm at distances of 1.5, 2.0, 2.5, and 3.0 m. 0° represents perpendicular aspect and error % is level of accuracy measured as the difference between known and estimated lengths. Data are presented as the mean ± SD. Positive values represent over-estimation and negative under-estimation.

2.4 Fish length measurement accuracy

Results from both sonar and stereo-video measurements of synthetic targets in paragraph 2.3 showed a high degree of measurement accuracy is achievable. This validated the calibration standard of both systems and also revealed optimal conditions from which to achieve the most accurate results when measuring fish, in particular orientation of the object to the stereo-video camera or sonar. From these results it was deemed that images would be most suitable for measurement when fish presented at an angle between 30° and 60° away from perpendicular to the sonar. Further to this, findings from studies on Chinook salmon (*Oncorhynchus tshawytscha*) by the Alaska Department of Fish and Game (personal correspondence KL Middlemiss and S. da Costa) suggest the highest level of sonar accuracy is obtained when fish present in an s-shape. Fish measurements from the stereo-video images are most accurate when fish are at a perpendicular aspect to the stereo-video camera to ensure that the head and tail of the fish are clearly visible.

Yellow-eyed mullet and snapper (n=20 each) were lightly anaesthetised individually (20 ppm AQUI-S), body length measured (fork length), and then each transferred into the previously described observation tank. Fish were then imaged singularly (i.e. one fish in the tank at a time) using stereo-video camera and sonar (approximately 2 min per fish to ensure the fish passage passed in front the camera and sonar systems frequently enough to allow measurement). On completion, fish were netted while still lightly anaesthetised and transferred back to their home tank. This was repeated for each fish. Using the aspect methods described above, estimated fish length was derived from sonar and stereo-video camera images (n=10 per fish from separate frames) and compared with known fish lengths.

Consistent with results from synthetic target measurements, fish length measurements of both yellow-eyed mullet and snapper using the stereo-video camera system were also

underestimates (Fig. 8a). Mean error rates for both species were around 5%. Interestingly, the sonar measurements of both species showed a mean error rate of almost nil for snapper compared with yellow-eyed mullet at around 9.5%. This may be indicative of the differences in swimming speed whereby snapper appeared to swim much slower (velocity not measured), or morphometrics that could have affected the clarity of the image produced by the sonar beams.

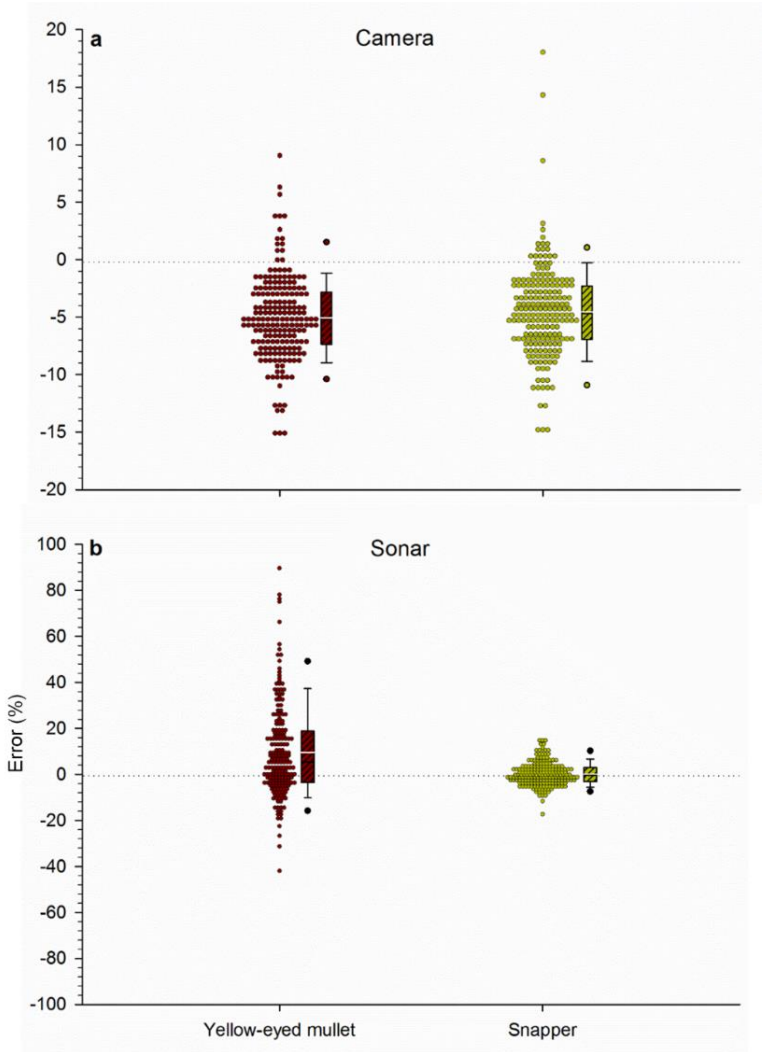

Figure 8 Measurement accuracy of yellow-eyed mullet (*Aldrichetta forsteri*), and snapper (*Chrysophrys auratus*) body length (fork length) calculated as mean percentage error from the ratio of difference between known and estimated lengths measured from stereo-video camera (a), and sonar (b) imagery. Data points for each species represents n=20 individual fish (n=10 measurements per fish). Positive values represent over-estimation of length, negative represent under-estimation. Box plots represent mean (white lines), and 5th and 95th percentiles.

2.5 Species identification from sonar morphometric measurements

To investigate the possibility of extracting morphometric characteristics from sonar images for use in species identification a mean ratio was calculated (total body length/height) from n=10 fish each of the three species listed in Table 2. Results showed that identification was possible for snapper, but not yellow-eyed mullet and kahawai given their similar morphometrics, therefore species identification from wild sonar footage (Chapter 8) required ground truthing with paired video footage.

Table 2 Ratio of height to total body length in yellow-eyed mullet (*Aldrichetta forsteri*), snapper (*Chrysophrys aurat*us), and kahawai (*Arripis trutta*).

	Yellow-eyed mullet	Snapper	Kahawai
Mean ratio height : length	4.4	3.4	4.2
Standard deviation	0.3	0.2	0.2

3 Do close neighbours become good friends? Behavioural interactions in mixed-species assemblages

3.1 Abstract

Marine ecosystems provide food, refuge and nursery habitat for fish species across all life-stages. Many species are opportunistic feeders, therefore dietary overlap is common and can result in competitive interactions for food resources, especially in geographically limited habitat such as estuaries. Yet this very same species richness, and often high abundance, can often provide food for higher trophic levels (e.g. avian predators). These trophic interactions and species dynamics likely influence why many fish adopt schooling behaviours which are considered to increase both individual foraging success and predatory avoidance capabilities. To investigate the behavioural dynamics of three sympatric species, tank-based behavioural studies were undertaken that presented species with feed competition and simulated predator attack scenarios. These species were kahawai (*Arripis trutta*), yellow-eyed mullet (*Aldrichetta forsteri*), both obligate schoolers, and snapper (*Chrysophrys auratus*), a facultative schooler. Stereo-video observations were made under four behavioural states: (1) control, (2) aerial predator, (3) diving predator and (4) feeding, while the behavioural variables of nearest neighbour distance, separation angle, swimming velocity, group size (area), species positioning and food consumption were determined. Kahawai and yellow-eyed mullet formed a single group displaying schooling behaviour while snapper formed a sub-group displaying shoaling behaviour. Kahawai outcompeted other species in foraging success (mean food consumption 75% of total consumed by all species), were more frequently positioned in the lead portion of the school (deemed a position of food dominance), and had varied swimming velocity compared with yellow-eyed mullet. Snapper showed less group cohesion due to shoaling behaviour. Results elucidate the complexities of interactive feeding and predatory behaviours in sympatric fish species.

3.2 Introduction

Marine ecosystems, and in particular the focus of the current study is on estuarine ecosystems, are complex habitats that are influenced by many abiotic and biotic factors including freshwater, tidal fluxes and saltmarshes and reed beds. (Pihl et al., 2002). Estuaries are considered to provide four primary functions to fish species: feeding, nursery (including juvenile life-stage refugia), spawning and diadromous migration pathways (Pihl et al., 2002). These highly productive ecosystems provide food for many species across several trophic levels right from consumers of benthic primary producers (e.g. algae and detritus) through the food chain to pelagic apex predators (Elliott & Hemingway, 2008; Pasquaud et al., 2010; Pihl et al., 2002). While estuaries may be considered productive at both the primary (e.g. microalgal) and secondary (e.g. teleostean) levels, predatory dynamics associated with higher (tertiary) trophic layers undoubtedly influence the behavioural and feeding dynamics of fish species that occupy estuary environments.

Estuarine habitats are considered some of the most resource rich on earth and globally teleosts represent high levels of species richness and abundance (biomass) within all trophic levels of these systems (Allen et al., 2006; Costa et al., 2002; Pasquaud et al., 2010; Potter et al., 2010). For example, estuarine species commonly include mullet, flatfish, herring, anchovy, cod and sparids (Costa et al., 2002), forming a large guild of fish that depend on these dynamic ecosystems for their survival (Potter et al., 2010; Potter et al., 2015). Fish occupying the same geographic area are highly likely to be exposed to competitive and opportunistic feeding interactions, both intra and interspecific (sympatry). Therefore, to reduce sympatric competition and improve individual fitness, they require a suite of species-specific foraging strategies. Mechanisms known to improve competitive foraging success in many species include physiological tolerances (Allen et al., 2006), opposing diurnal feeding rhythms (Blaber,

1976), and collective behaviours (e.g. schooling) (Partridge, 1982). Fish are known to form both mono- and multi-species aggregations (Lukoschek & McCormick, 2002); however, little is known of the competitive foraging pressures within the latter.

Estuarine ecosystems are commonly considered safe havens for fish species, particularly within the nursery context for juvenile life-stages (Pihl et al., 2002), but this is a contentious research area (Baker & Sheaves, 2007). Refuge is considered only as a secondary function of nursery habitat use in studies by Pihl et al. (2002), whereby the inhabitation of shallow estuarine water by fish is considered to enable fish to escape piscivorous predation (Paterson & Whitfield, 2000). Aquatic predation, while arguably more common, is not the only predation risk and the effects of avian foragers on fish behaviour has gained very little research effort to date (Cruz et al., 2015). By way of example of the risks posed by birds, shallower waters could be considered more accessible to piscivorous avian predators including the very common and widely distributed shag (*Phalacrocorax sp.*) (Cosolo et al., 2010; Paulin & Paul, 2006). Therefore, aerial predation poses a considerable risk to fish survival, as was shown by extensive capture and mortality of sticklebacks, and a sparidae species, by shorebirds within various estuarine environments (Blaber, 1973; Whoriskey & Fitzgerald, 1985). Given these examples of substantial aerial predation of fish in estuarine systems, the argument that estuaries can provide predator refuge may not be true in all contexts, adding doubt to the estuarine refuge hypothesis. However, similarly to foraging competition, collective behaviour is also a mechanism used by fish to reduce predation risk (Pitcher, 1998); therefore, collective behaviours are likely associated with feeding competition and predator avoidance, within the estuarine context.

Yellow-eyed mullet (*Aldrichetta forsteri*), kahawai (*Arripis trutta*) and snapper (*Chrysophrys auratus*) are three such sympatric species commonly found occupying temperate estuaries and inshore waters of New Zealand and Australia (Ghasemzadeh, 2016; Morrison et al., 2014).

Yellow-eyed mullet are an often abundant, obligate schooling species, and considered resident estuary opportunists (Jones et al., 1996). They utilise these ecosystems for both their rich food sources and nurseries (including refuge) for vulnerable juvenile life stages (Curtis & Shima, 2005; Jones et al., 1996). An opportunist detritivorous species (Cardona, 2015), yellow-eyed mullet range between benthic and pelagic habitats feeding mainly on organic matter found in sediment, algae, crustaceans and to a much lesser degree small fish (i.e. short-finned eel (*Anguilla australis*), inanga (*Galaxias maculatus attenuatus*) and common smelt (*Retropinna anisodon*) (McMillan, 1961; Webb, 1973b). This broad diet and low trophic positioning likely contributes to the ability of yellow-eyed mullet to be such an abundant estuarine species (Edgar & Shaw, 1995; Whitfield et al., 2012).

Snapper and kahawai, are also ontogenetically associated with estuaries utilising them as nursery habitat as juveniles, but then migrating to deeper coastal waters at sub-adult life stages and returning seasonally to feed in the productive inshore and estuarine waters (Baker, 1971; Griggs et al., 1998; Morrison et al., 2014; Parsons et al., 2016; Parsons et al., 2014). Kahawai are opportunistic feeders with a diet of crustaceans and small fish, which at older life stages includes yellow-eyed mullet (Baker, 1971; Morrison et al., 2014). Estuarine associated snapper are demersal benthic grazers, feeding mainly on crustaceans, shellfish, but also fish (Colman, 1972; Godfriaux, 1969; Morrison et al., 2014). In summary, dietary overlaps exist for all three species (particularly in juvenile life-stages when foraging crustaceans) (Morrison et al., 2014), they are natural prey of pied shag *Phalacrocorax varius* in New Zealand estuaries (personal observation), are all known to respond to supplementary feeding in the wild (Middlemiss et al., 2017a), and show similar collective behaviours in estuaries.

There are many complexities associated with relationships in estuarine fish and few authors have analysed the behavioural responses generated by competitive interactions, including foraging and predation risk, in species occupying these marine dynamic environments. Whilst

the focus of the current study is placed in an estuarine context, findings are relevant to and can be applied to a much broader application of general fish behavioural interactions in all ecosystems. We investigated interactions among three gregarious, sympatric fish species under conditions of foraging competition, and a simulated predation risk, to determine how each cohabiting species performs during competitive interactions, and identify how collective behaviours for each species enhances individual fitness in sympatric systems.

3.3 Materials and methods

3.3.1 Experimental animals

Three fish species: kahawai, yellow-eyed mullet and snapper, were used in tank-based experiments conducted at The New Zealand Institute for Plant & Food Research Limited (PFR) Seafood Research Facility in Nelson, New Zealand. Fish were held in aerated 5000 L flow through tanks, using filtered seawater pumped ~30 L min^{-1} from the surrounding Nelson Haven and water chemistry maintained at ~pH 7.6, 35–36 PSU and DO >90%. Mean nominal ambient seawater temperature over the study period (January–February 2016) was 22.4 °C SD ± 0.9. Tank stocking densities were maintained at 15–25 kg^{-1} m^3 and fish were twice daily fed a Skretting® diet (Nova ME, Skretting, Australia) calculated at 2% body mass. Kahawai and yellow-eyed mullet (obligate schoolers), were wild caught from the Nelson Haven (41.254°S, 173.278°E) in December 2014 and 2015 respectively, and snapper (facultative schoolers) were randomly sampled from a population of reared fish, spawned from wild sourced broodstock held at the PFR facility. A total of 30 fish per species were separated into n=3 groups consisting of 10 fish of each species (i.e. group 1 = 10 each of kahawai, yellow-eyed mullet and snapper, etc.). All fish were used only once to avoid any possible conditioning effect on results. Individuals of each species were of comparable body mass and fork length (FL) where mean

(± SD) mass and FL for all 30 fish in each species were: (1) yellow-eyed mullet 255 ± 5 mm, 226 ± 18 g, (2) kahawai 235 ± 10 mm, 204 ± 25 g and (3) snapper 270 ± 9 mm, 457 ± 45 g.

3.3.2 Experimental tank setup

Experiments were carried in a circular 13,000 L tank (diameter 3.5 m, depth 2.6 m) using filtered seawater (1 μm to maximise water clarity) pumped from the surrounding Nelson Haven (Fig. 1). To standardise experimental conditions between fish, food was withheld 24 h prior to experiments, flow was turned off during the observation periods, and immediately prior to experimental work beginning animals were lightly anaesthetised (20 ppm, AQUI-S®) then transferred from home tanks into the experimental tank and given 24 h to acclimate. Apparatus included a feeding tube positioned over the centre of the tank (pellets administered ~15 times at 30 s intervals), a horizontal pulley system (~1 m above the water surface) used to present a simulated avian aerial and diving predator models (generic seabird at full wingspan), and a shroud to partition the observer from fish during experiments. Three dimensional (3D) imagery was acquired using a stereo-video camera system (consisting of two Go-Pro® cameras (1080 p resolution, 60 frames per second, medium field of view (FOV)) attached to a base plate with a lens separation distance of 420 mm at a 4° inwards incline each camera). The camera system was affixed to the tank wall at a depth of ~1 m. A light-emitting diode (LED) was used twice (3–5 sec interval) at the beginning of recordings to synchronise cameras and a standardised start time of ~1000 h was observed for all observations (one replicate per day).

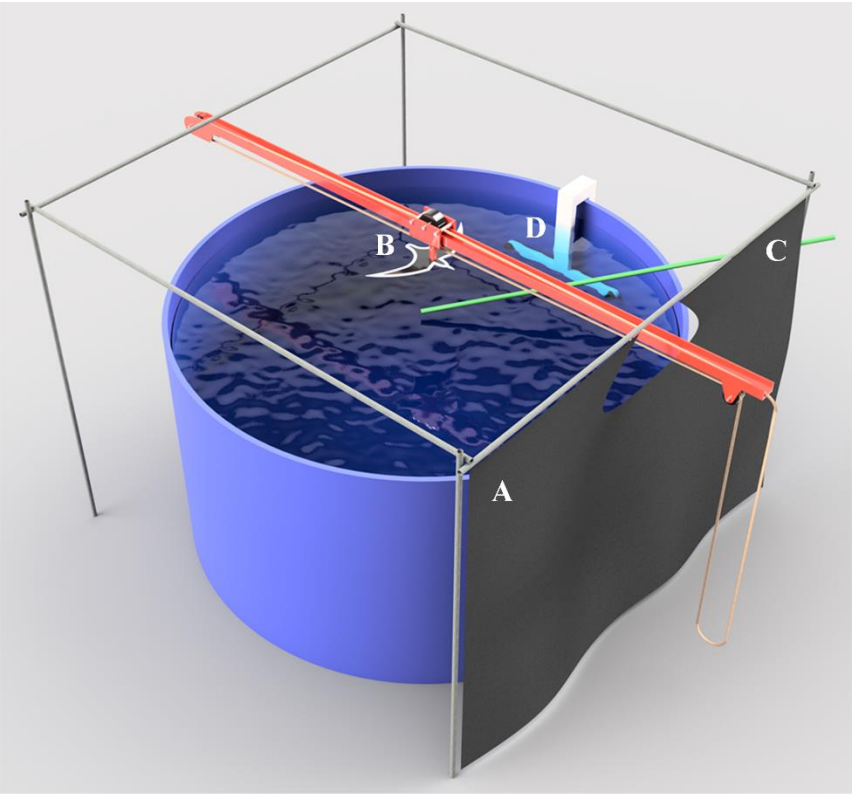

Figure 1 Schematic of experimental tank setup showing (A) shroud to hide observer from fish view, (B) pulley system for simulated alarm response using avian aerial and diving predators, (C) feeding tube and (D) stereo-video camera system. Image credit: Olly Burrow.

3.3.3 Behavioural manipulations

Fish were observed under the following behavioural states: (1) control, (2) aerial predator, (3) diving predator, and (4) feeding. Observations were conducted over a continual 50 min period and video footage divided into the following segments: (a) 0–10 min (acclimation period once camera system placed in tank), (b) 10–20 min (control), (c) 20–30 min (aerial predator), (d) 30–40 min (diving predator) and (e) 40-50 min (feeding). This was standardised for all replicates. For the aerial predator exposures (c) the previously described avian predator model was pulled across the tank and back ~20 times at ~30 s intervals; the return passage took ~3–5 s. The diving predator exposure (d) was presented by dropping the bird model into the tank then immediately removing it at ~30 s intervals. Feeding behaviours (e) were investigated using

the same commercial pellet feed used for daily rearing (providing continuity) and introduced in ~5 g amounts into the tank via the feeding tube.

3.3.4 Behavioural measurements

Variables of nearest neighbour distance (NND), separation angle (SA) and swimming velocity, commonly used to quantify school structure (Masuda & Tsukamoto, 1998; Santana-Garcon et al., 2014), were analysed during each of the four behavioural states (b–e). Measurements were made from stereo-video footage using SeaGIS EventMeasure® software (SeaGIS, 2017). Briefly, the software measured NND from the 3D mid-point of each fish (3D mid-point is calculated from the mean of 3D head and tail positions (FL)), SA from the angle between the directions of each pair of fish (fish direction defined by the 3D head and tail coordinates), and swimming velocity from 3D head positions measured at different time points (i.e. t_1 & t_2). Fish were only selected for measurement when the head and tail were clearly visible in each frame. The NND, SA and swimming velocity were computed for each individual (swimming velocity n=5, NND and SA n=10 each species), then a mean was calculated using all individual measures made over n=3 replicate frames (~30 s apart) and repeated for each of the three replicate mixed-species groups. During aerial and diving predator, and feeding behavioural states, fish were measured ~5 s after the event to standardise measurement conditions between replicates. Snapper were measured for SA in the control state only, where occasional schooling behaviour was displayed. However, during other behavioural states a more definite shoaling behaviour was displayed, which meant head and tail were not clearly visible and fish vectors (calculated from head and tail coordinates) could not be accurately determined for a sufficient number of individuals. Because of this, for aerial and diving predator and feeding behavioural states snapper NND was measured using 3D point measurements from the head position only (rather than the 3D mid-point) and therefore no SA could be generated.

Group size (area m^2) was calculated from a 2D irregular polygon using x, y coordinates from vertices around the periphery of the group applied to the following formula:

$$\text{Area} = (x1\ y2 - y1\ x2) + (x2\ y3 - y2\ x3) \ldots + (xn\ y1 - yn\ x1) \div 2$$

Where $x1$ is the x coordinate of vertex 1 and yn is the y coordinate of the n^{th} with the final term repeating the first to finish at the starting vertex. In mixed-species schools, the proportion of each species (kahawai and yellow-eyed mullet) within the lead half of the school (i.e. first 10 fish) was calculated from n=9 measurements (3 x repeated measures from n=3 mixed species groups). Repeated measures of the first 10 fish in each of the three replicates were obtained from camera frames separated by one complete swimming rotation of the tank. Food consumption by individual species was calculated as the mean percentage from n=3 mixed species replicates by dividing total pellet consumption (per species) by total number of pellets offered per feeding (15 consecutive feeds over ~7 min for each replicate). Only pellets consumed whilst suspended in the water column (i.e. not from the tank floor) and within the camera field of view were counted. On completion of observations, all fish were transferred back to the main holding tank.

3.3.5 Statistical analysis

Data was analysed using R v.3.3.2, SigmaPlot v.12.5 and Genstat v.17, and are represented as means ± 95% confidence interval (CI) unless otherwise stated. Schooling data NND, SA and swimming velocity were analysed in R using a linear mixed model (LMM) with restricted likelihood to account for the unbalanced nature of the data using the asreml-R library. Response variables failing to meet assumptions of constant variability and normality were log-transformed and behaviour, fish size and their interactions were fitted as fixed-effects and replicate groups as a random effect. Food consumption was analysed using a Poisson generalized linear mixed model (LMM) (Genstat) and a log link with factors for each feeding

event (n=15), within each rep (n=3) for each species (n=3) and for the uneaten portion. Interaction terms tested whether there were linear or quadratic trends in the proportion of food eaten by each species and the differences between replicate in the species effect and trends. Positioning of individual species within a mixed-species school was analysed using a binomial generalized linear model/logistic regression (LLR) (Genstat), and group size (area m^2) using a 2-way ANOVA (SigmaPlot) with species and behavioural state as fixed effects. Pairwise comparisons (Tukey test) were used to analyse statistically significant interactive effects between species and behavioural state (group size). Significance was accepted at $P \leq 0.05$.

3.4 Results

3.4.1 Qualitative behavioural observations

Two separate groupings formed from the three species used in experiments: (1) kahawai (*Arripis trutta*) and yellow-eyed mullet (*Aldrichetta forsteri*) amalgamated into one school and (2) snapper (*Chrysophrys auratus*) remained in a distinctly separate sub-group. These behaviours were maintained under all behavioural states (control, aerial predator, diving predator and feeding) and there were no visible signs of aggression among species or conspecifics. Each group occupied a separate level in the water column: snapper predominantly shoaling beneath the kahawai/yellow-eyed mullet, which schooled above them in a continuous clockwise direction. During the control period, snapper displayed occasional short bouts of cohesive schooling behaviour, utilised the whole tank area, and generally appeared more coordinated in their shoaling behaviour (in all replicate groups). However, they became a more loosely aligned shoal and appeared more disrupted during all other behavioural states. Immediate alarm responses in snapper during simulated aerial and diving predators included occasional displays of cryptic behaviour by some individuals, including motionlessness and camouflage tactics, as well as much tighter groupings towards the floor of the tank.

Kahawai/yellow-eyed mullet initially showed reduced swimming velocity and occasionally the lead half of the school would double back to reform a tighter packing density within the group before continuing on schooling continuously. Immediate threat responses appeared diminished with decreased exposure and during foraging the snapper returned to behaviour more similar to that shown in the control period.

3.4.2 Quantitative analysis of school structure

3.4.2.1 NND, SA and swimming velocity

Means for all NND, SA and swimming velocity data are displayed in Table 1. The unit of measure for NND and swimming velocity in Figures 2 and 4 are given in both mm and BL; however, all analysis is discussed in terms of BL to avoid duplication, unless otherwise stated.

There was a significant interactive effect of species and behavioural state on NND (LMM; Wald test statistic = 27.9, $DF = 3$, $P < 0.001$) (Fig. 2, Table 1). Inter-individual spacing in the snapper control group was significantly increased in comparison to the kahawai/yellow-eyed mullet control (~0.2 BLs, LMM; Wald test statistic, = 2.585, $DF = 1$, $P = 0.026$). Within group comparisons showed that kahawai/yellow-eyed mullet NND was higher during feeding (~0.2 BLs) compared with the control (LMM; Wald test statistic = 24.145, $DF = 3$, $P = 0.023$). In the snapper group, NND decreased significantly during the diving predator alarm response (~0.2 BLs) compared with feeding (LMM; Wald test statistic = 24.145, $DF = 3$, $P = 0.013$), however not compared with aerial or diving predator behavioural states ($P = 0.066$ and $P = 0.054$ respectively). Between group comparisons of kahawai/yellow-eyed mullet and snapper showed significant differences between corresponding controls (increased NND in snapper) and aerial diving (decreased NND in snapper) predator behavioural states (LMM; Wald test statistic = 2.585, $DF = 1$, $P \leq 0.05$ for both).

Table 1 Measurements of group structure in kahawai *(Arripis trutta)*, yellow-eyed mullet (YEM) (*Aldrichetta forsteri*) and snapper (*Chrysophrys auratus*). Measurement variables include nearest neighbour distance (NND), separation angle (SA) and swimming velocity under four behavioural states; control, aerial and diving predators and feeding. NM, not measured. Data represented as means ± 95% CI.

Measurement variables	Species	Behavioural state			
		Control	Aerial predator	Diving predator	Feeding
NND (BL)	Kahawai/YEM	0.8 ± 0.03	0.8 ± 0.04	0.9 ± 0.04	1.0 ± 0.1
	Snapper	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.1
SA (°)	Kahawai/YEM	14.9 ± 1.6	16.6 ± 1.6	17.0 ± 2.6	19.6 ± 2.2
	Snapper	22.4 ± 3.1	NM	NM	NM
Velocity (BL s^{-1})	Kahawai	3.2 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	3.8 ± 0.2
	YEM	2.9 ± .1	2.8 ± 0.1	2.9 ± 0.2	3.8 ± 0.1
	Snapper	1.5 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1

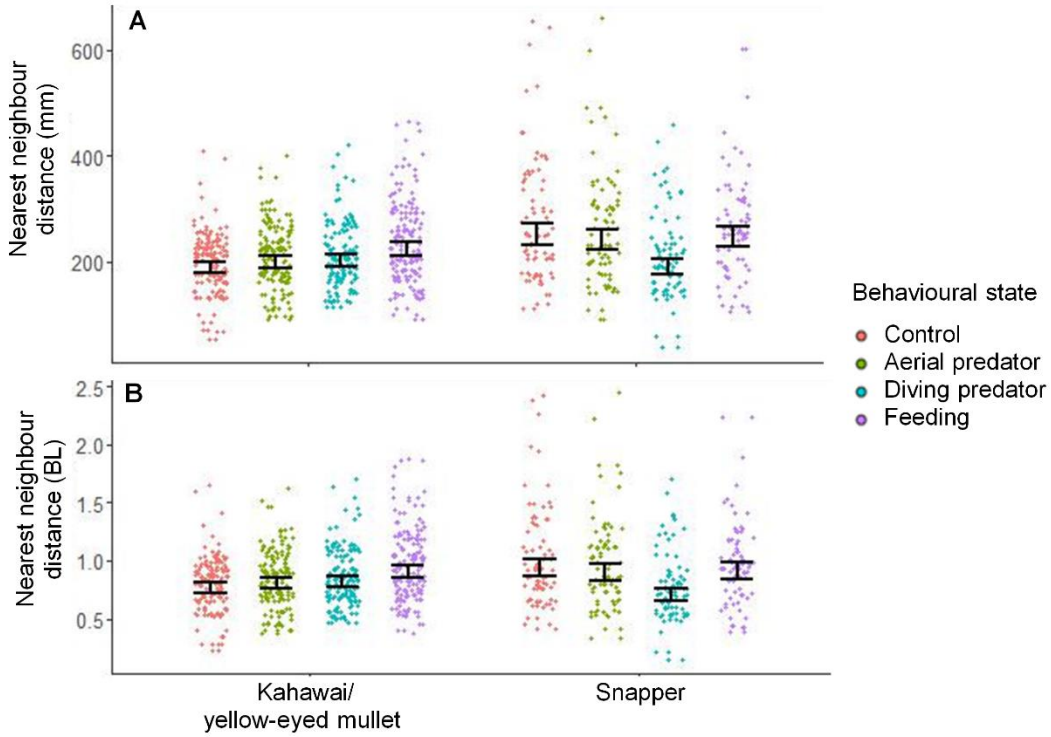

Figure 2 Nearest neighbour distances (A: mm, B: body length (BL, fork length)) between mixed-species groups of kahawai (*Arripis trutta*) and yellow-eyed mullet (*Aldrichetta forsteri*), and mono-species groups of snapper (*Chrysophrys auratus*) during control, aerial predator, diving predator and feeding behavioural states. Dots represent individual fish and error bars are 95% CI. If no overlap exists the difference between groups is considered significant with significance accepted at $P \leq 0.05$.

Comparison of mean SA within the kahawai/yellow-eyed mullet group (Fig. 3, Table 1) showed no significant difference between control vs aerial predator and diving predator feeding behavioural states (LMM; Wald test statistic = 11.831, *DF* = 3, *P* > 0.05 for all). However, fish were significantly less aligned whilst feeding in comparison to the control group (LMM; Wald test statistic = 11.831, *DF* = 3, *P* = 0.050). Snapper SA in the control group was significantly higher (~30%) than the kahawai/yellow-eyed mullet control (LMM; Wald test statistic = 18.27, *DF* = 1, *P* < 0.001).

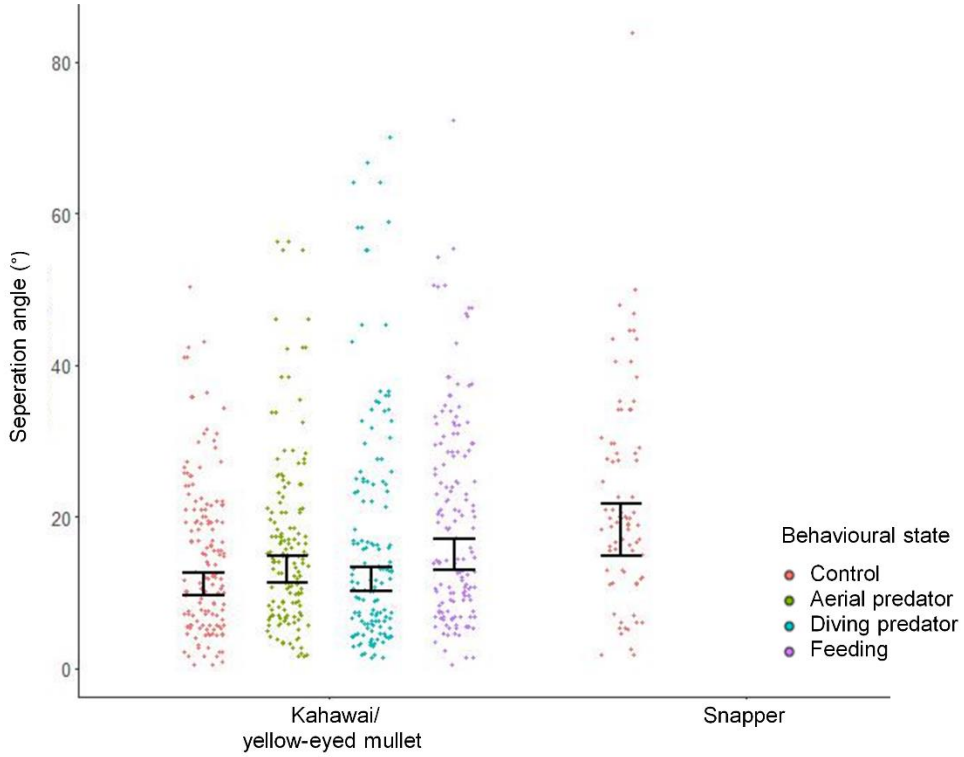

Figure 3 Mean separation angles between mixed-species groups of kahawai (*Arripis trutta*) and yellow-eyed mullet (*Aldrichetta forsteri*), and mono-species groups of snapper (*Chrysophrys auratus*) during control, aerial predator, diving predator and feeding behavioural states. Dots represent individual fish and error bars are 95% CI. If no overlap exists the difference between groups is considered significant, with significance accepted at $P \leq 0.05$.

Swimming velocity was identified for all three species, as opposed to sub-groups (i.e. kahawai/yellow-eyed mullet and snapper). Within group comparison for snapper (Fig. 4, Table

1) showed significantly decreased swimming velocity (between ~20% and 33%) in aerial predator, diving predator and feeding behavioural states compared with the control group (LMM; Wald test statistic = 179.745, *DF* = 3, $P < 0.001$ for all). Snapper swam significantly slower (LMM; Wald test statistic = 3586.135, *DF* = 2, $P < 0.001$ for all) by between 2- and 3-fold in comparison with kahawai/yellow-eyed mullet, during all behavioural states. During feeding both kahawai and yellow-eyed mullet had significantly higher swimming velocities (~19% and 31%) compared with controls (LMM; Wald test statistic = 179.745, *DF* = 3, $P = 0.004$ and $P = 0.001$ respectively). Within the kahawai group, this trend was reversed during the aerial response with significantly reduced swimming velocity by around 9% when compared with the control group (LMM; Wald test statistic = 179.745, *DF* = 3, $P = 0.006$). Interestingly, during the aerial predator response, absolute swimming velocity (mm s^{-1}, Fig. 4a) for individual species in the mixed-species kahawai/yellow-eyed mullet group showed a significantly reduced velocity in kahawai by ~12.5% (LMM; Wald test statistic = 3011.15, *DF* = 2, $P = 0.009$).

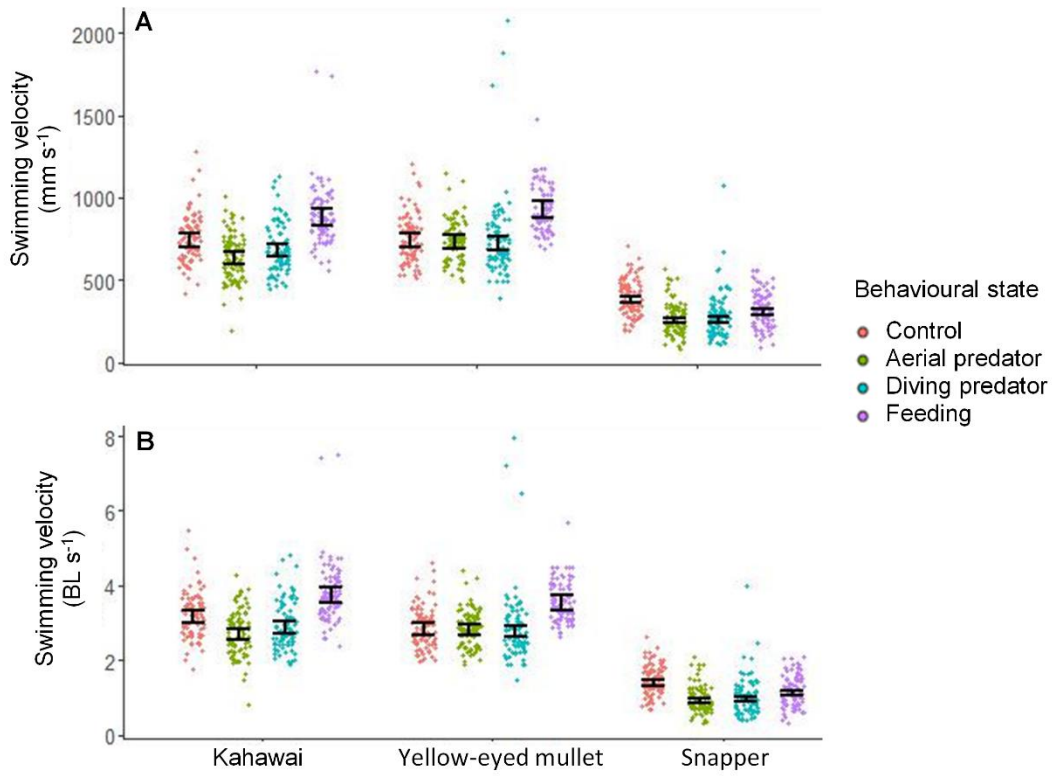

Figure 4 Swimming velocity expressed in mm s^{-1} (A) and body lengths (BL, fork length, B) of kahawai (*Arripis trutta*), yellow-eyed mullet (*Aldrichetta forsteri*), and snapper (*Chrysophrys auratus*) during control, aerial predator, diving predator and feeding behavioural states. Points represent individual fish and error bars are 95% CI. If no overlap exists the difference between groups is considered significant, with significance accepted at $P \leq 0.05$.

3.4.2.2 Group size (area), lead school composition and food consumption

Analyses of changes in size (area m^2) for fish groups, during different behavioural states, were restricted to within group comparisons of kahawai/yellow-eyed mullet (n=20 fish) and snapper (n=10 fish) given the unbalanced number of fish in each group. During feeding, kahawai/yellow-eyed mullet mean area (0.53 ± SD 0.14 m^2) was significantly larger by ~32% and ~56% compared to means for aerial (0.40 ± SD 0.07 m^2, ANOVA; Tukey's test, $q = 7.03$, residual $DF = 64$, $P < 0.001$) and diving (0.34 ± SD 0.05 m^2, ANOVA; Tukey's test, $q = 4.77$, residual $DF = 64$, $P = 0.007$) predator responses, but not different to the control (ANOVA; Tukey's test, $q = 3.557$, residual $DF = 64$, $P = 0.067$) (Fig. 5). In snapper there was a significant

decrease in group size during aerial (0.20 ± SD 0.04 m^2, ANOVA; Tukey's test, q = 4.32, residual DF = 64, P = 0.017), diving predator (0.15 ± SD 0.04 m^2, ANOVA; Tukey's test, q = 6.24, residual DF = 64, P < 0.001) and feeding (mean 0.22 ± SD 0.03 m^2, ANOVA; Tukey's test, q = 3.73, residual DF = 64, P < 0.05) behavioural states compared with the control (0.32 ± SD 0.07 m^2).

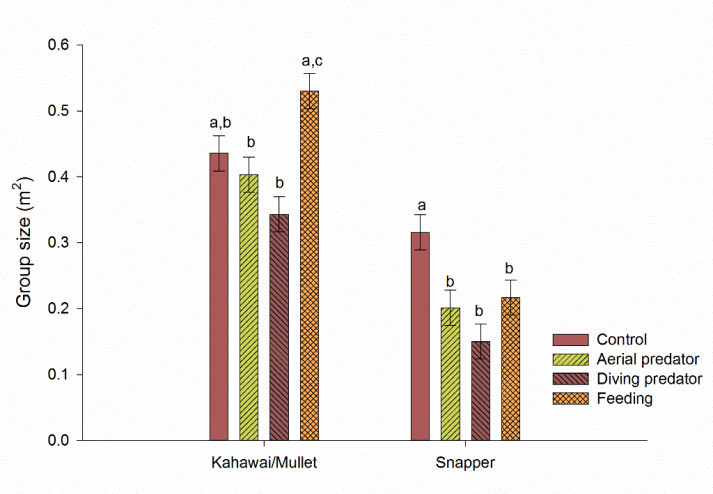

Figure 5 Changes in group size (area m^2) of mixed-species groups of kahawai (*Arripis trutta*) & yellow-eyed mullet (*Aldrichetta forsteri*), and mono-species groups of snapper (*Chrysophrys auratus*) during control, aerial predator, diving predator and feeding behavioural states. Error bars represent means ± SD. Significant differences within both fish groups represented by different letters (a–c) and significance accepted at $P \leq 0.05$.

Kahawai spent significantly more time in the lead half of the school than yellow-eyed mullet, under all behavioural states (control, aerial predator, diving predator, and feeding) (LLR; Wald tests comparing kahawai means on logit scale vs 0 (50%) 2.09 to 5.56, P = 0.0366 to < 0.0001) (Fig. 6). Control means in both kahawai and yellow-eyed mullet were 61.1 ± SD 14.5%, and 38.9 ± SD 14.5%. The lowest occupancy rate for the lead portion of the school by yellow-eyed mullet was during the diving predator alarm response (mean 17.8 ± SD 4.4%). During an alarm response from a diving predator, kahawai spent significantly more time in the lead half of the school (~34%) than during control or feeding states (LLR; deviance = 14.39, DF = 3, P = 0.0024). Within the yellow-eyed mullet group, occupation rates in the lead half of the school

were significantly reduced by ~54% in the diving predator state compared to the control (LLR; deviance = 14.39, *DF* = 3, *P* = 0.0024).

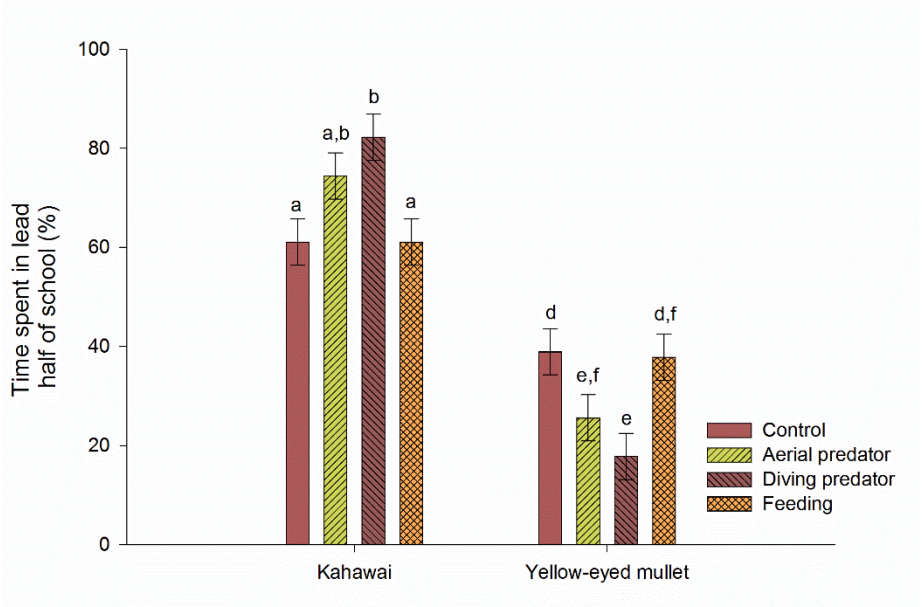

Figure 6 Species composition of lead half (first 10 fish) of mixed-species schools of kahawai *(Arripis trutta)* and yellow-eyed mullet *(Aldrichetta forsteri*) under differing behavioural states. Data are represented as means ± SD. Significant differences represented by letters a-e and significance accepted at $P \leq 0.05$.

During feeding, kahawai consumed an average of 75% (CI ± 9.8%) of the combined food consumed in the water column (excluding floor portion) by all three species over 15 consecutive feeding periods (Fig. 7). This was significantly more than either yellow-eyed mullet (15.3 ± CI 7.3%) or snapper (7.5 ± CI 5.3%) (LMM; *F* = 12.3, *DF* = 1 and 3.6, *P* = 0.0295). Yellow-eyed mullet and snapper did not differ significantly in the amounts consumed (LMM; *F* = 0.7, *DF* = 1 and 4.4, *P* = 0.4340). In two of the three replicates the proportion of food eaten by kahawai declined in later feedings, but the pattern was not consistent enough to be significant (LMM; *F* = 3.7, *DF* = 1 and 3.6, *P* = 0.1346).

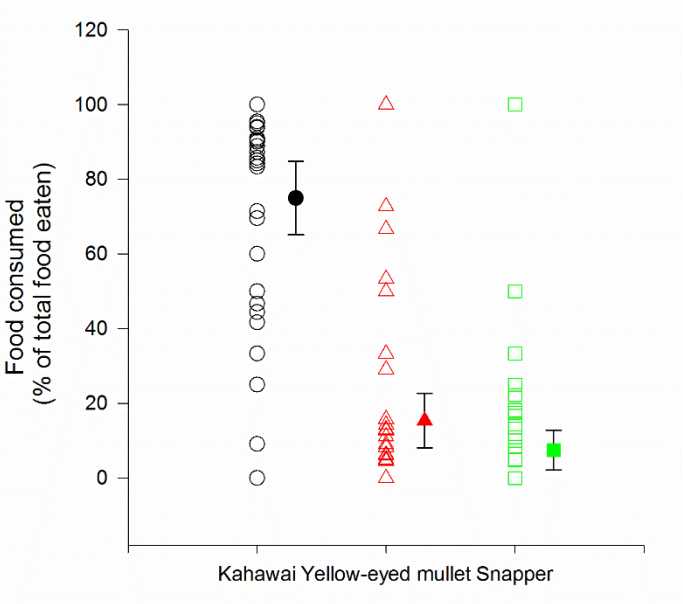

Figure 7 Mean percentage of food consumed by each species of the total eaten by mixed-species groups of yellow-eyed mullet *(Aldrichetta forsteri)*, kahawai *(Arripis trutta)* and snapper *(Chrysophrys auratus)*. Data points represent individual feeding events (n=45) and error bars 95% CI.

3.5 Discussion

3.5.1 Qualitative interspecific behaviours

It is not uncommon for mixed-species aggregations of fish with similar phenotypic characteristics to form in the wild (Krause et al., 1996b; Pitcher, 1986), and this clearly applied in the current experiment where yellow-eyed mullet and kahawai formed a single school. Kahawai are a natural predator of yellow-eyed mullet in the wild (Baker, 1971), therefore, it is interesting that these two species formed a single group and that no signs of aggressive behaviour were observed. It is assumed that limited time in the experimental tank (~26 h) in combination with similar phenotypic characteristics (e.g. comparable body size and the insufficient gape in kahawai), contributed to passive behaviour. It is not entirely surprising that snapper did not school with kahawai/yellow-eyed mullet given their very different phenotypic characteristics, which dictate lesser athletic capacities in the laterally compressed snapper.

Other mitigating factors may also include hatchery reared (snapper) vs wild (yellow-eyed mullet and kahawai) fish having differing learned behaviours. Further comparative studies could be performed to identify if the presence of snapper intrinsically contributed to the combined schooling behaviour seen in kahawai and yellow-eyed mullet.

Schooling behaviour was displayed consistently throughout in kahawai/yellow-eyed mullet, whereas snapper predominantly shoaled (although control fish did display occasional schooling behaviour). Both occupied different areas of the water column, but displayed different behaviours in immediate response to predator threat and foraging, with some snapper initially displaying cryptic behaviours, and the group increased packing density and took up occupation near the tank floor, before normal swimming resumed shortly after the initial response. Alternatively, kahawai/yellow-eyed mullet showed an initial lowering of swimming velocity and occasional group reformation before resuming normal swimming behaviour. It is likely that due to continuous schooling, it was necessary for the kahawai/yellow-eyed mullet to take the higher vertical position in the water column to avoid collision, forcing snapper lower down into the tank and resulting in snapper employing cryptic behaviours.

It is interesting that threat response appeared be become habituated with exposure to predation threat (results not presented), perhaps due to only a perceived, rather than real risk being incorporated into our experimental design. Similarly, reduced responses were found in guppies (*Poecilia reticulata*) (Vanesyan et al., 2015) after repeated startle responses, and the authors' personal observations of yellow-eyed mullet predator-prey interactions in the wild during avian predation (pied shag) is that this species quickly returns to prior behaviour (i.e. foraging) after the initial response. Our tank-based studies suggest both kahawai and yellow-eyed mullet may possess differing threat response strengths that are directly attributable to the number of exposures. This may be an important behavioural adaptation to reduce the time spent on redundant anti-predator behaviour (Vanesyan et al., 2015), after repeated responses with no

negative conclusion, allowing that time to instead be spent on other fitness increasing activities such as foraging. Quantification of this would be an interesting area of further research.

Of further interest, a partitioning or hierarchy within the school structure appears to exist within kahawai/yellow-eyed mullet (likely related to food consumption rates) with kahawai represented in the lead section between 60 and 80% of the time, across all behavioural states. Results investigating competitive advantages between the three species (competing for the same food resource) also found that kahawai were the dominant foragers consuming 75% of available food. We suggest that on formation of multi-species assemblages the food dominant species adopt positions within the school that best advantage foraging success, which conceivably would lead to faster growth and increased fitness. This is supported by previous studies on positioning behaviour within fish groups reviewed in Krause (1993). The question can then be asked as to what advantage yellow-eyed mullet gained from forming a school with kahawai when foraging success was so greatly reduced. Given two of the principal benefits of schooling behaviour, are decreased predation risk and increased foraging success (Partridge, 1982), our results suggest that predation risk likely plays a role in group formation and outweighs the negative effects associated with food competition by other group members.

3.5.2 Quantitative analysis of school structure

In addition to qualitatively describing interspecific behaviours, we also quantitatively measured changes in group structure (NND, SA and velocity) during feeding and simulated predator attacks. As expected, we found disrupted behaviour as was similarly found in the effects of an aerial predator on shoaling in golden shiner (*Notemigonus crysoleucas*) (Litvak, 1993). However, our results also showed varied responses among species and it is reasonably assumed that these were due to differences in swimming behaviour with snapper predominantly

forming un-cohesive shoal structures and kahawai/yellow-eyed mullet forming much more cohesive and aligned school formations as detailed below.

3.5.2.1 Nearest neighbour distance

Within the kahawai/yellow-eyed mullet group NND remained relatively similar during aerial predator and diving predator behavioural states compared with the control group. Yet group structure was significantly disturbed during feeding with distances between fish increasing by ~16% in comparison with the control fish. It is reasonable to assume that as foraging urgency increased, interindividual space also increased as a strategy to augment improved feeding success rates. A similar theory was suggested from findings in bluntnose minnows (*Pimephales notatus*) (Morgan, 1988). Within the snapper, there was a significant decrease in NND during the diving predator state of ~20% in comparison with the control state, but only limited differences between the aerial predator and control states. It is likely that the threat to snapper posed by the diving predator (but not to the aerial predator) highlights differences in avoidance/anti-predator strategies employed by benthic associated species (e.g. stationary and camouflage tactics), and/or may relate to previously mentioned adaptive behaviours associated with cultured (snapper) vs wild caught (kahawai and yellow-eyed mullet) fish. Between group comparisons showed snapper NND was ~20% larger in the control and lower in the aerial predator behavioural states than the kahawai/yellow-eyed mullet under the same conditions. It is assumed that this is directly attributable to differences in schooling vs shoaling behaviour displayed by both groups.

3.5.2.2 Separation angle

School structure was further quantified in terms of cohesiveness, measured as polarity and characterised by SAs between fish (0° separation = parallel orientation). Kahawai/yellow-eyed mullet showed sustained group cohesiveness under control, aerial predator and diving predator

behavioural states, except during feeding, where similarly to results from NND, group behaviour was disrupted with increased SA (~24%). Studies of alignment in groups of guppies (*Poecilia reticulata*), after a startle response, found a positive correlation between increased polarisation and decreased NND (Vanesyan et al., 2015). It is suggested that increased alignment of individuals assists with maintenance of increased swimming velocity by the group as a whole as part of the behavioural response to a predator attack (Viscido et al., 2004). However, our results show very little variation (range of ~4°) in polarisation within kahawai/yellow-eyed mullet under any behavioural state. Perhaps this indicates a persistent level of heightened awareness among individuals as a consequence of these two species schooling together. Between group comparisons of SA in control fish found snapper were less aligned than kahawai/yellow-eyed mullet (~33%). This was expected given the different swimming behaviours between groups (i.e. schooling v shoaling), and because schools consist of highly polarised individuals, whereas shoals are loosely formed aggregates (Pitcher, 1983, 1998).

3.5.2.3 Swimming velocity

Lastly, we measured changes in swimming velocity in all three species. Results showed that during feeding, kahawai and yellow-eyed mullet swimming velocity increased by ~19% and ~31% in comparison with the control group, possibly indicating increased urgency whilst foraging. However, snapper showed reduced swimming velocities of around 20% in response to the same event when compared with control fish. The difference in snapper behaviour could be a result of the increase in foraging activity in the other species causing snapper to become apprehensive. As expected, swimming velocity in shoaling snapper was ~50% slower than schooling kahawai or yellow-eyed mullet in control groups. Interestingly, given they formed a mixed-species school, kahawai, during the aerial and diving predator alarm response, decreased absolute swimming velocity by ~12.5% and ~8%, compared with yellow-eyed mullet, which

maintained similar rates across control, aerial predator and diving predator states. This highlights a level of disruption existing within the mixed-species school. It is surprising given that schooling requires unified behaviours (Larsson, 2012), including swimming velocity; however, this may be as a result of phenotypic differences between species. This disruption within the group may suggest a reason why mixed-species schools are less common in the wild than mono-species assemblages. A major contributing factor to maintenance of group cohesiveness in schools is that constant adjustments are made between individuals to avoid contact with each other (Katz et al., 2011). In addition to this, it appears that choosing to swim with a different species, although of a phenotypically similar body shape, adds a secondary layer to maintenance of cohesiveness, because of differences in swimming behaviour. In this example, participation in mixed-species schooling behaviour may compromise optimisation of swimming speed, ultimately affecting individual fitness. It is not known if the decision to form a mixed-school was mutual between species and if not, what the costs (e.g. energetic) are to the more dominant group. This would be an interesting area for future research effort.

3.5.3 Estuarine context

Our study is of particular relevance to understanding multi-species behavioural interactions within highly dynamic estuarine ecosystems. The finite amount of geographical space associated with estuaries, combined with limited food resources and multi-species co-habitation, likely results in high levels of interspecific competition, particularly during foraging. Considering just competition, it is likely that a feeding hierarchy exists among species in shared habitat with some species being more dominant. Our results from tank-based investigations of three New Zealand estuarine species have elucidated possible behavioural interactions experienced by these fish in wild populations. Snapper, perhaps due to displaying predominantly shoaling behaviour, did not interact with other schooling species. Interestingly, kahawai, although natural predators of yellow-eyed mullet (Baker, 1971), chose to school

together, perhaps because of similar body size and obligate schooling behaviour. As previously mentioned, it is not uncommon for fish to form mixed-species groupings (Krause et al., 1996b; Pitcher, 1986), but it is not known if this exists in natural populations, although our results suggest it is possible given the right environmental circumstances and that fish perhaps have a larger suite of behaviours than we may realise.

Despite their cohabitation and overlapping resource use, some variation in the eco-physiological traits of these three species is apparent. Notably, morphological differences exist between these species with snapper body shape being laterally compressed, which varies greatly to both kahawai and yellow-eyed mullet which possess a fusiform shape (Froese & Pauly, 2017). Whether these morphological characteristics and associated swimming capacities and hydrodynamic characteristics influence fish behaviours and competitive interactions in multi-species assemblages (e.g. foraging success) is not well known.

However, within the mixed-species group a definite feeding dominance existed in favour of kahawai, as evidenced by frequent positioning of this species in the lead portion of the school, and outcompeting others during foraging. It is possible that the trade-off for decreased foraging success experienced by yellow-eyed mullet during the initial stages of a foraging event is balanced by reduced predation risk associated with increased group membership. It is plausible that given yellow-eyed mullet seemed incapable of outcompeting kahawai in a mixed-species assemblage that they could also utilise alternative feeding strategies that associate with morphological and behavioural differences related to their species specific foraging ecology (e.g. differences in feeding apparatus, diurnal patterns, etc.). This could potentially create a partitioning of resource use (i.e. fish who attack food at the water surface as seen in kahawai, and those whose feeding strategy include benthic foraging (e.g. snapper)). This is a theory supported by research on prey selection in shallow water sympatric species *Mullus barbatus*, *Mullus surmuletus, Serranus cabrilla* and *Serranus hepatus* (Labropoulou & Eleftheriou,

1997). Such morphological differences may have been a contributing factor in the current results where depth partitioning in the water column was observed. It would be interesting to see how species react under less controlled conditions (i.e. fish of different size, total abundance/biomass).

3.6 Summary

Tank based studies in mixed-species groups of kahawai, yellow-eyed mullet and snapper were investigated for behavioural interactions. Phenotypically similar kahawai and yellow-eyed mullet schooled cohesively and continually under all behavioural states, but, showed differences in swimming velocity. Snapper formed a sub-group with reduced group cohesion and swimming velocity due to shoaling behaviour. Overall, kahawai were the dominant species, outcompeting others in foraging ability, which is likely due to differences in phenotypic and behavioural repertoires. Results also showed that mixed-species schools displayed more dominant behaviour than a mono-species sub-group. It is likely that the intraspecific behavioural strategies displayed in our tank based experiments exist in natural environments; however, further research is required to confirm this theory. Our research provides valuable insight in to the complexity of feeding and predatory behaviours particularly in estuarine fish species, but also has wider implications for improving our general understanding of behavioural interactions of fish in all ecosystems.

3.7 Ethics statement

All experiments were conducted in accordance with the University of Canterbury Animal Ethics Committee (Ref: 2014/35R). Methods were carried out in accordance with approved guidelines.

4 Effects of group size on school structure and behaviour in yellow-eyed mullet (*Aldrichetta forsteri*)

4.1 Abstract

Group living is a phenomenon found in many animal species and around 50% of fish display this behavioural trait. Individuals conforming to a set of group interaction rules contribute to coordination in fish aggregates. The dynamic nature of estuarine ecosystems requires fishes to adapt to daily challenges. These include frequent contact with other species (sympatric populations of resident and migratory fish), resulting in increased competition for food resources and predator-prey interactions. Identifying spatial patterns that arise from interaction rules associated with group formation is essential to our understanding of collective behaviour in sympatric fish populations within marine environments. Tank-based three-dimensional studies of yellow-eyed mullet (*Aldrichetta forsteri*) schools (15, 75 and 150 individuals) investigated the effects of group size on school structures during predation risk and foraging states. Increasing group size correlated to increased nearest neighbour distance. Swimming velocity was variable between groups and was the lowest in groups of 15 fish. Immediate alarm response strategies, resulting from the simulated avian predator, differed between size groups. School shape was an oblong/oblate spheroid with a ratio of 5:2:1, and the area of free-space surrounding individual fish was spherical in shape with a high degree of spatial isotropy present in all size groups. Our results challenge traditional theories that it is either/or local and global properties that are the key drivers in maintenance of small group structure, and suggest a broader collaborative approach is at play in mechanisms used for collective behaviours in these fishes.

4.2 Introduction

Collective behaviour is a widespread phenomenon seen within the animal kingdom; however, no other vertebrate taxa displays a greater range of collective behaviour than fish (Vicsek & Zafeiris, 2012), which possess the highest level of diversity with ~30,000 catalogued species (Eschmeyer et al., 2010). Social living improves individual fitness by conferring advantages

such as increased foraging success and decreased predation risk (Partridge, 1982), and fish schools come in all shapes and sizes (Pavlov & Kasumyan, 2000). The seemingly effortless coordination witnessed in groups of fish (Couzin et al., 2002; Krause & Ruxton, 2002; Partridge, 1982) requires each individual to match the movement of its neighbour instantly, using a suite of sensory capabilities (e.g. lateral line system (Middlemiss et al., 2017b), resulting in the whole group travelling together as one cohesive unit. The singular appearance of fish groups in various shapes (e.g. oblong and ellipsoid) conceals the constant state of deliberate self-organisation (Breder, 1954), where each individual continuously responds and conforms to group consensus, to maintain a singular and cohesive entity (Paramo et al., 2010; Partridge, 1980; Pitcher & Partridge, 1979; Sumpter et al., 2008). This evolutionary survival strategy (Ballerini et al., 2008) requires a number of behavioural traits to be employed by individuals.

Properties thought to shape coordinated schooling behaviour can be categorised as local (individual interactions) or global (e.g. aggregated school size), and both have received considerable research effort, as discussed in Rieucau et al. (2015a). Localised behaviours are guided by a well-defined set of interaction rules (e.g. nearest neighbour distance (NND) and separation angle (SA)), which help to regulate inter-individual behavioural interactions (Herbert-Read, 2016; Rieucau et al., 2015a; Tien et al., 2004). Each individual applies these rules in response to sensory cues (e.g. visual and mechanosensory (Bleckmann, 1986)) generated by changes in immediate neighbour behaviour when responding to external stimuli (e.g. predator-prey interactions and foraging) (Herbert-Read, 2016). With regards to global properties that shape schooling behaviour, there have been many discussions around the existence of optimal group sizes and the dispersal of knowledge through large groups (Krause & Ruxton, 2002; Pulliam & Caraco, 1984). Kunz and Hemelrijk (2012) suggest that a maximum group size exists, beyond which individuals are no longer influenced by global

properties because they can no longer perceive the group as a whole. Arguments have been made that theoretically even if there were an optimal group size, it would be seemingly unattainable given the cognitive decisions that would be required by every fish to maintain a maximum number of individuals within any group (Pulliam & Caraco, 1984; Rieucau et al., 2015a).

Whilst the effects of both group size and individual interactions have been well studied, there is ongoing debate as to which of the two is the leading contributor to maintenance of group structure in schooling fish. As examples, Rieucau et al. (2015a) describes the impact of localised interaction rules in relation to the information transfer and behaviours of immediate neighbours (e.g. NND and SA) in ensuring maintenance of a cohesive group structure, and similarly Ballerini et al. (2008) argue that it is the number of individuals in the group with which each individual interacts, rather than group size per se. As safety and foraging success are key motivations for schooling behaviour, an understanding of individual interaction rules in the context of different size fish groups, when faced with predator and foraging responses, will help us to better understand the factors which shape wild schooling behaviour.

Yellow-eyed mullet are an abundant estuarine species found commonly in temperate and tropical waters of New Zealand and Australia (Curtis & Shima, 2005; Morrison et al., 2014; Paulin & Paul, 2006). Estuarine habitats are dynamic (e.g. daily migration of fishes in and out of estuaries with tidal flow), with overlapping trophic layers which result in increased predator-prey interactions and competition for resources (e.g. space and food) in sympatric estuarine environments (Whitfield, 2015). Yellow-eyed mullet, and the wider Mugilidae family, typically school in large numbers within estuarine habitats (Crosetti & Blaber, 2016) in groups of various sizes. Therefore, it is plausible that yellow-eyed mullet schools undergo daily changes to group membership as a result of possible migration of individuals between groups

(e.g. sub-groups joining other groups, or splitting of larger aggregations) because of biotic and abiotic factors.

Using controlled experiments, we identified the impact that group size had on interaction rules and decision making (local and global properties) which affect yellow-eyed mullet school structure during similar behavioural states (predation threat and foraging) experienced by wild populations. Variables of interest included the initial behavioural response to predator threat, NND, SA, swimming velocity, packing density (free-space around individuals) and morphological change to school shape. In describing changes in school structure, we hypothesised that individuals would swim closer together in response to a predator threat and further apart when foraging. We propose that combined local and global interaction rules are intrinsically involved in the maintenance of school structure, and that both these rules drive behaviours which enhance schooling behaviour in fish populations.

4.3 Materials and methods

4.3.1 Experimental animals

Tank-based experiments were undertaken at The New Zealand Institute for Plant & Food Research Limited (PFR) seafood research facility in Nelson, New Zealand, during July 2015. Groups of yellow-eyed mullet (*Aldrichetta forsteri*) were caught from the Nelson Haven estuary (41.254°S, 173.278°E) in December 2014 and reared in aerated 5000 L flow-through tanks, using filtered seawater pumped at ~30 L min^{-1} from the surrounding Nelson Haven. Water chemistry was maintained at dissolved oxygen (DO) >90%, ~pH 7.6, and 35–36 practical salinity units (PSU). A diet of Skretting® (Nova ME, Skretting, Australia) was fed twice daily calculated at 2% body mass per day and tank stocking densities did not exceed 15–25 kg m^3. During the study period mean (± SD) nominal ambient seawater temperature was 10.2 °C ± 0.9. A total of 720 fish were sub-sampled into three size groups: 15, 75 and 150 fish

(n=3 replicates per group) and each fish was used once to avoid any possible conditioning effect. Mean mass (g) and length (fork length, FL) (± SD) for the total number of fish in each group were: (1) 15; 83.5 ± 3.9 g, 186.4 ± 2.7 mm, (2) 75; 80.0 ± 1.9 g, 183.2 ± 1.9 mm, and (3) 150; 84.8 ± 2.7 g, 186.0 ± 1.7 mm.

4.3.2 Experimental tank setup

Observations were carried out in a 13,000 L flow-through tank (diameter 3.5 m, depth 2.6 m), using filtered (1 μm) seawater. Flow was ceased during observation periods, to standardise conditions between fish groups. Animals were lightly anaesthetised (20 ppm, AQUI-S®) prior to transfer from home tanks into the experimental tank and given ~24 h to acclimate prior to experimental work beginning. Food was withheld for ~24 h prior to experiments for all groups. Tank setup (Fig. 1) consisted of a feeding tube centrally positioned over the tank, from which food pellets were administered ~15 times at 30 s intervals. An alarm response was elicited using a simulated avian aerial predator pulled across the tank and back (via a horizontal pulley system positioned ~1 m above the water surface) ~20 times at ~30 s intervals, each with a ~3-5 s exposure. One complete swimming rotation of the tank was allowed between exposures. Image acquisition was obtained in three dimensions (3D) using a calibrated stereo-video camera system consisting of two Go-Pro® cameras (1080 p resolution, 60 frames per second, medium field of view (FOV)) with a lens separation distance of 420 mm at a 4° inwards incline (each camera), giving a stereo overlap of ~3.8 m (horizontal) and ~2.3 m (vertical) at a range of 3.5 m. Cameras were synchronised using a flashing light twice (3–5 sec interval) at the beginning of recordings and then attached to the tank wall (~1 m below the water surface). All observations had a standardised start time of ~1000 h (one fish group per day). The observer was concealed behind a shroud during experiments.

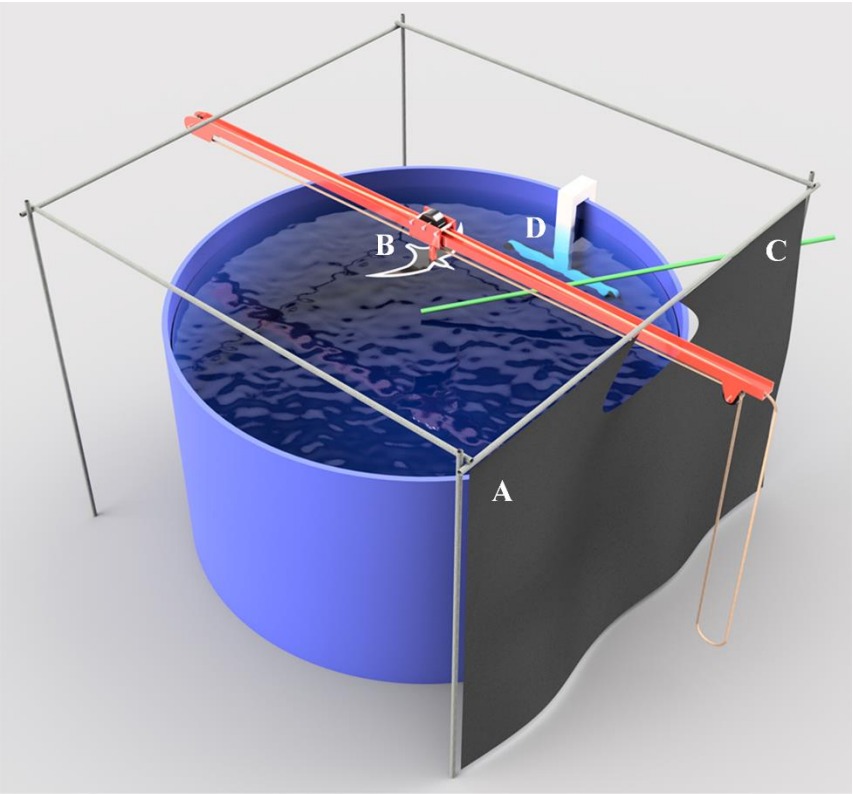

Figure 1 Representation of experimental tank setup showing (A) shroud, (B) pulley system for simulated avian alarm response, (C) feeding tube, and (D) stereo-video camera system. Image credit: Olly Burrow.

4.3.3 School structure measurements

Fish groups (sizes 15, 75 and 150) were observed under control, aerial predator and feeding behavioural states. Stereo-video footage observation periods consisted of a continuous 40 min period divided into: (1) 0-10 min (acclimation period once camera system placed in tank), (2) 10-20 min (control), (3) 20-30 min (aerial predator), and (4) 30-40 min (feeding). Measurements were based on n=3 replicate groups for each size group (except size group 150, n=2) and n=3 repeat measures (separated by a minimum of one complete swimming rotation of the tank) within each size group and behavioural state. Fish were exposed to treatments (aerial predator and feeding), then after completing one tank rotation, measurements were taken as soon as the group came back into the camera FOV. The number of fish visible for NND and SA measurement was limited to the FOV and therefore, numbers of individuals measured

varied depending on the size group. In respect of this, all fish were sampled in size group 15 for NND and SA measurements, and sub-sampling was done for size groups 75 and 150 fish (~30-40 fish in each). For swimming speed a mean was calculated using all individual measures made from n=5 fish over n=3 replicate frames (one tank swimming rotation apart) and repeated for each of the three replicates in each size group. For NND, SA and swimming speed, measurements were made from stereo-video footage using SeaGIS EventMeasure software (SeaGIS, 2017). NND was measured from the 3D mid-point calculations for each fish, being the mean of the 3D head and tail positions. The angle between the directions of each pair of fish (direction characterised from the 3D head and tail coordinates) was used to calculate SA, and swimming velocity was calculated from the 3D head positions at different time points (i.e. t_1 and t_2). The mean 3D free space (packing density) around each fish was calculated by: (1) distance (mm) from a focal fish to the nearest fish in directions front, behind, above, below, right and left) and (2) median distance in x, y and z planes as BL^3. If no fish was present (i.e. focal fish had no other fish above it), no value was recorded. Only fish clearly visible (head and tail) in each frame were selected for measurement. Behavioural responses to an aerial predator were measured from exposures (n=10) in each of the three replicates in each size group. School morphology (span in metres of the length, breadth and height based on x, y, z coordinates of all measured individuals) was quantified from three repeat measures in each replicate group in size group 15, as this was the only group with all fish visible in the camera FOV.

4.3.4 Statistical analysis

Analysis was carried out using Rv. 3.3.1 (NND and SA) and SigmaPlot v.12.5 (swimming velocity and packing density). NND was analysed using a linear mixed model and for SA a generalised linear mixed model with a log-link function was fitted; both with group size and behaviour as fixed effects and replicates as random effects. Swimming velocity and packing

density data were analysed using 2-way ANOVA (factors size group and behavioural state) and Tukey's LSD test post hoc for pairwise multiple comparisons. School morphology was analysed using 1-way ANOVA to compare means of school length, breadth and height. Data are given as means ± 95% CI unless stated otherwise and if CI error bars do not overlap the difference between two groups can be deemed significant. Significance was accepted at $P \leq 0.05$.

4.4 Results

4.4.1 Nearest neighbour distance, separation angle and swimming velocity

All fish groups displayed obligate schooling behaviour throughout experiments and maintained continuous clockwise rotational swimming. Means for all results in this section are displayed in Table 1. Group size and behavioural state had a significant effect on NND (LMM; Wald statistic 338.6 and 160.7, $DF = 2$, $P < 0.001$ both) (Fig. 2A, Table 1). Increases in control group NND means were positively correlated with group sizes 15, 75 and 150 and during an alarm response to a simulated aerial predator threat NND was reduced in all three size groups compared with controls. The greatest reduction in NND between control and aerial predator was seen in size group 150 (~56 mm or ~24%). During feeding behaviour, mean NND increased compared with distances shown during the aerial predator threat in size groups 15, 75 and 150. However, this difference was only significant in size group 150 (LMM; Wald statistic, 141.1, $DF = 2$, $P < 0.001$) with an increase of around 78 mm (~43% or ~0.4 BL). Between size-group comparisons of mean NND during feeding showed that size group 15 was significantly smaller than size groups 75 or 150 (LMM; Wald statistic 72.4, $DF = 1$, $P < 0.001$ respectively), and size group 75 was not significantly smaller than size group 150 (LMM; Wald statistic 12.3, $DF = 1$, $P = 0.051$). Comparisons between control and feeding behaviour showed

no significant difference in NND in size groups 15, 75 or 150 (LMM; Wald statistic 1.37, *DF* = 1, *P* = 0.2413).

Table 1 Measurements of yellow-eyed mullet nearest neighbour distance (NND), separation angle (SA) and swimming velocity in size groups 15, 75 and 150 fish during control, aerial predator and feeding behavioural states. Data represented as means ± 95% CIs.

Group size	Measurement variable	Behavioural state		
		Control	Aerial predator	Feeding
15	NND (mm)	193.7 ± 10.7	139.7 ± 7.3	181.3 ± 10.0
	SA (°)	16.5 ± 1.9	17.6 2.2	19.4 ± 2.6
	Velocity (BL s^{-1})	2.8 ± 0.3	2.7 ± 0.4	2.7 ± 0.2
75	NND (mm)	228.5 ± 8.2	183.5 ± 6.0	240.7 ± 10.8
	SA (°)	17.2 ± 1.6	19.6 ± 1.6	20.5 ± 2.0
	Velocity (BL s^{-1})	3.7 ± 0.1	4.0 ± 0.3	3.7 ± 0.1
150	NND (mm)	235.1 ± 10.6	178.7 ± 7.0	259.9 ± 12.1
	SA (°)	18.0 ± 1.7	17.6 ± 1.7	17.5 ± 2.0
	Velocity (BL s^{-1})	3.5 ± 0.2	3.4 ± 0.3	3.7 ± 0.1

Mean SA ranged between 16.5° and 20.5° for all size groups and behavioural states (Table 1, Fig. 2B). Group size had a significant effect on SA (LMM; Wald statistic 15.09, *DF* = 2, *P* = 0.0005); however, differences were not strongly biological relevant and only appear statistically significant because of large sample sizes. No significant differences were found between the mean values between behavioural states within each size group or (*P* = 0.083).

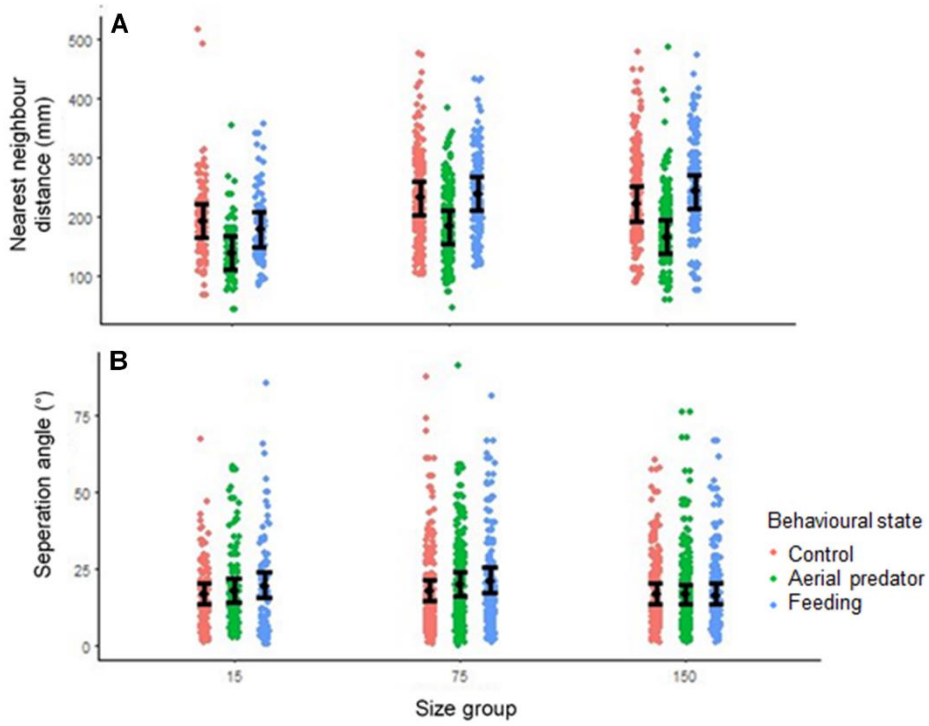

Figure 2 Nearest neighbour distances (A) and separation angles (B) in size groups 15, 75 and 150 of yellow-eyed mullet (*Aldrichetta forsteri*) during control, aerial predator and feeding behavioural states. Dots represent raw data and error bars are 95% CI. Significance was accepted at $P \leq 0.05$.

Comparisons of mean values for swimming velocity in control, aerial predator and feeding behavioural states, within each of the three size groups (Table 1, Fig. 3), found no significant difference (ANOVA; $F = 0.163$, $DF = 2$, $P = 0.849$), and no significant interactive effect between behavioural state and group size (ANOVA; $F = 1.70$, $DF = 4$, $P = 0.149$). However, between-group comparisons (15, 75 and 150 individuals) showed swimming velocity was significantly lower in size group 15 (~1 BL s^{-1}) during all behavioural states in comparison to size groups 75 and 150 (ANOVA; $F = 105.87$, $DF = 1$, $P < 0.001$ for all). There was also a significant increase in mean swimming velocity (~18%) in size group 75 compared with size group 150 during the aerial predator alarm response ($P < 0.001$).

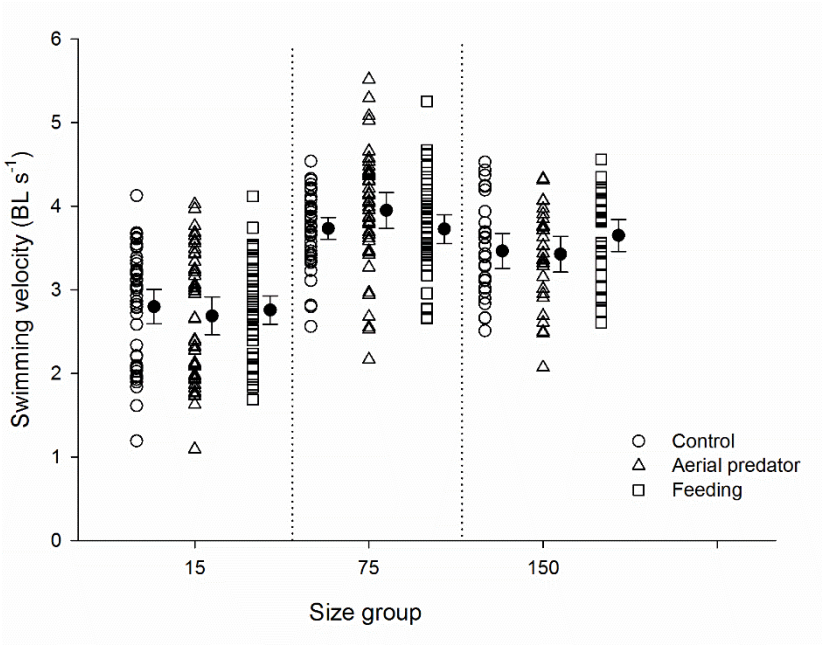

Figure 3 Swimming velocity in size groups 15, 75 and 150 of yellow-eyed mullet (*Aldrichetta forsteri*) during control, aerial predator and feeding behavioural states. Points represent raw data and error bars are 95% CI. Significance was accepted at $P \leq 0.05$.

4.4.2 Packing density and school morphology

Packing density within fish schools was quantified by the amount of free-space surrounding individual fish. Results are given as volume (BL^3) (Table 2), and mean NND distances (mm) in six spatial directions (i.e. front, behind, above, below, left and right), along x, y and z planes (Fig. 4). Volume was calculated using the following equation described in Pitcher and Partridge (1979):

$$\left(\frac{4}{3}\right) * \pi * x\ y\ z\ /\ 2$$

where x, y and z are median distances (BLs) in x, y and z planes (e.g. x: median of the sum of all distances in positions front and back as shown in Fig. 4). Within each of the three size groups, individuals displayed consistent mean NND (mm) in all spatial directions (isotropic spacing), regardless of behavioural state. Therefore, the envelope of free-space around each fish was evenly spaced (Fig. 4) and can be described as spherical. However, the volume (BL^3)

of free-space around individuals in size group 15 was between 2–3 fold smaller than in both larger size groups under all behavioural states (Table 2).

Table 2 Estimated volume of free-space (BL^3) around individual yellow-eyed mullet (*Aldrichetta forsteri*) in school sizes 15, 75 and 150 during control, aerial predator and feeding behavioural states based on median nearest neighbour distance (NND) in x, y and z planes. Volume calculated as (4/3 * π * x y z) / 2. Data represented as mean (± SE).

Group size	Plane	Median NND (BL)			Volume (BL^3)		
		Control	Aerial predator	Feeding	Control	Aerial predator	Feeding
15	x	1.12 ± 0.02	0.82 ± 0.01	1.08 ± 0.02	-	-	-
	y	1.16 ± 0.03	0.83 ± 0.03	1.10 ± 0.03	-	-	-
	z	1.15 ± 0.04	0.83 ± 0.03	1.09 ± 0.04	3.13	1.18	2.71
75	x	1.43 ± 0.02	1.18 ± 0.02	1.44 ± 0.03	-	-	-
	y	1.44 ± 0.03	1.17 ± 0.02	1.43 ± 0.03	-	-	-
	z	1.42 ± 0.03	1.20 ± 0.02	1.46 ± 0.04	6.12	3.46	6.29
150	x	1.46 ± 0.02	1.12 ± 0.02	1.63 ± 0.03	-	-	-
	y	1.51 ± 0.03	1.14 ± 0.02	1.63 ± 0.03	-	-	-
	z	1.48 ± 0.03	1.13 ± 0.04	1.61 ± 0.05	6.83	3.02	8.95

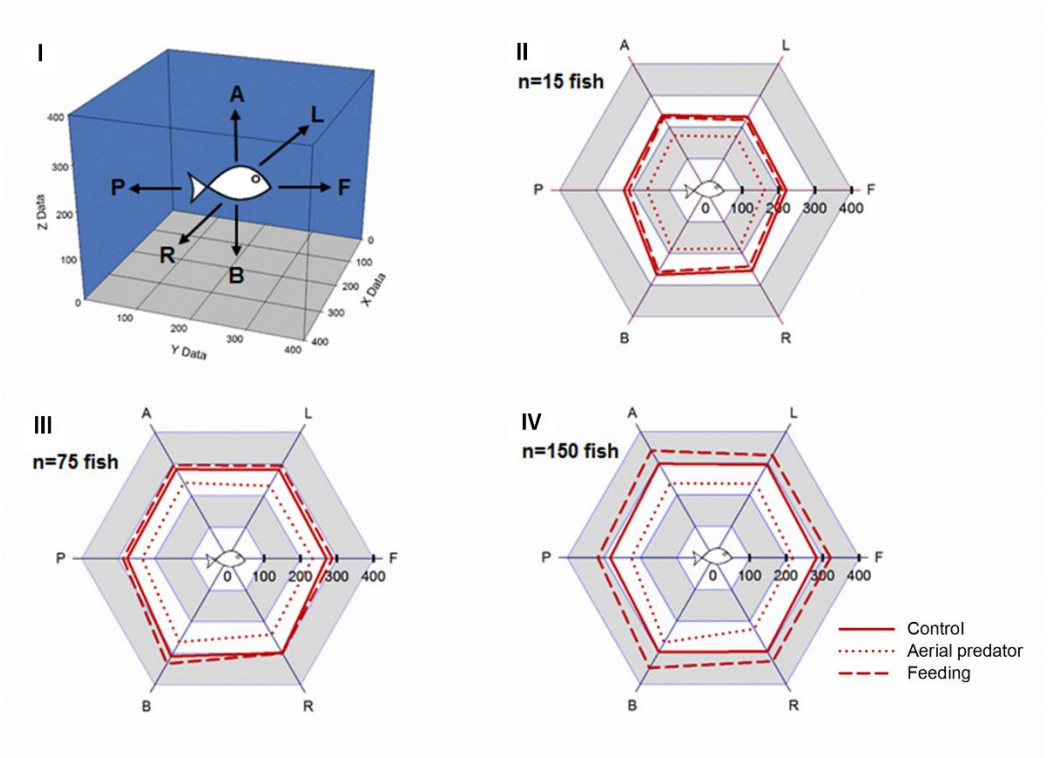

Figure 4 Schematic representing the mean three-dimensional (3D) free-space (I) around individual fish in schools of yellow-eyed mullet (*Aldrichetta forsteri*) in size groups 15 (II), 75 (III) and 150 (IV) under control, aerial predator and feeding behavioural states. Space represented by distance (0-400 mm) to the nearest fish in directions front (F), behind (P), above (A), below (B), left (L) and right (R) from the focal fish (centre of image).

Statistical analyses of mean distances (mm) in directions front, back, left, right, above and below in fish size groups 15, 75 and 150 during behavioural states control, aerial predator and feeding are summarised with *p*-values in Table 3. Similar spacing in all directions was found in control groups of the two larger size groups 75 and 150 (mean ~300 mm, Fig. 4 III-IV). However, there was significantly less spacing between size group 15 (Fig. 4 II) in comparison to both size groups 75 and 150, in all directions and during all behavioural states, except to fish directly below in size groups 15 vs 150. Distances in size group 15 compared with both size groups 75 and 150 during control and aerial predator behavioural states were smaller by around 23%. During feeding behaviour, NND increased significantly in all directions in size groups 75 and 150 compared with that in size group 15.

Table 3 Summary of significance tests (2-way ANOVA) between distances (mm) described in Figure 4 for yellow-eyed mullet (*Aldrichetta forsteri*) in size groups 15 vs 75 and 150, in six directions (above, below, back, front, left and right), during control, aerial predator and feeding behavioural states. Numbers indicate *p*-values with significance accepted at $P \leq 0.05$.

	Spatial direction					
	Above	Below	Back	Front	Left	Right
Size group			**Control**			
15 vs 75	0.003	0.004	<0.001	<0.001	<0.001	0.002
15 vs 150	<0.001	0.069	<0.001	<0.001	<0.001	0.005
75 vs 150	0.360	0.567	0.806	0.491	0.515	0.989
			Aerial predator			
15 vs 75	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
15 vs 150	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
75 vs 150	0.871	0.906	0.428	0.209	0.861	0.143
			Feeding			
15 vs 75	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
15 vs150	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
75 vs 150	0.007	0.529	0.104	0.042	0.094	0.127
Behavioural state			**15**			
Control vs aerial predator	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Control vs feeding	0.861	0.714	0.577	0.678	0.779	0.538
Aerial predator vs feeding	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
			75			
Control vs aerial predator	<0.001	<0.001	<0.001	0.001	<0.001	<0.001
Control vs feeding	0.513	0.104	0.589	0.349	0.572	0.991
Aerial predator vs feeding	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
			150			
Control vs aerial predator	<0.001	0.031	<0.001	<0.001	<0.001	<0.001
Control vs feeding	0.006	<0.001	0.032	0.012	0.073	0.053
Aerial predator vs feeding	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

The morphology (shape) of actively schooling groups of 15 mullet (n=3 groups) was calculated from the mean 3D span (m) from fish in the furthest extents of the school in x (length), y (breadth) and z (height) planes. Schools consisted of an oblong-type shape (e.g. ellipsoid/oblate spheroid) with mean distances of ~1.75 ± 0.30 m (x-axis), 0.59 ± 0.13 m (y-axis) and 0.33 ± 0.05 m (z-axis). This equates to a ratio of 5:2:1 (length, breadth and height respectively). Pairwise comparisons (Tukey's LSD test) of each axis length (x, y, and z) found significant differences between: length vs height $P < 0.001$, length vs breadth $P < 0.001$, and breadth vs height $P = 0.049$. In addition to describing the span of the schools in metres, using the volume of free-space calculated in Table 2 around each individual in schools of 15 fish (3.13 BL^3, mean BL = 186.4 mm), the cubic volume of space occupied by all individuals was estimated as 46.95 BL^3.

For comparison purposes, the spatial differences seen between fish in tank vs natural environment (Fig. 5) has been included and is an image taken of a slow-moving school of wild juvenile (size assumption based on approximate mean length of <200 mm) yellow-eyed mullet in a shallow (~1 m) estuary at Richardson Stream, Onetahuti Bay, Able Tasman National Park, New Zealand (40°52'53.6"S 173°03'04.5"E) (KL Middlemiss, pers. obs.). These fish, occupying shallow waters and albeit in a larger group size, appeared to display a larger ratio of breadth to length than results from our tank-based study, but still appeared in an oblate-spheroid shape.

Figure 5 Wild juvenile yellow-eyed mullet (*Aldrichetta forsteri*) schooling in a shallow, sandy, brackish estuarine environment, in the shape of an oblate-spheroid. Image taken in January 2016 directly above the school (~4-5 m) from a bridge crossing Richardson Stream in Onetahuti Beach, Able Tasman National Park, New Zealand (40°52'53.6"S 173°03'04.5"E). Photo credit: Karen L. Middlemiss.

4.4.3 Behavioural response to predator threat

The immediate post-alarm response to an aerial predator threat, in the smallest groups of fish (n=15), was to maintain polarised behaviour and swimming direction (Table 4). However, observed behaviour from stereo-video footage (change in acceleration not measured) clearly showed swimming velocity and group morphology were both momentarily reduced in response to the predator threat (Fig. 6). The behaviour of the larger groups (75 and 150) differed from that of size group 15, with school structure becoming disrupted, as represented in Figure 7 when the leading fish turned back on themselves, resulting in positional reorganisation and directional change for some individuals, and causing the group initially to become depolarised.

Table 4. Mean (± SD) percentage time yellow-eyed mullet (*Aldrichetta forsteri*) schools maintained polarised (P) or depolarised (DP) behaviour in response to an aerial predator threat. Significance accepted at $P \leq 0.05$ and bold numbers represent significant differences for pairwise comparisons between size groups within each behaviour state (i.e. P or DP).

Size group	School structure		Within size group *p*-value
	P (%)	DP (%)	P vs DP
15	**83.3** ± 11.5	**16.7** ± 11.5	< 0.001
75	6.7 ± 5.8	93.3 ± 5.8	< 0.001
150	16.7 ± 11.5	83.3 ± 11.5	< 0.001

P = group polarity maintained
DP = group became depolarised with individuals changing position and direction within the school

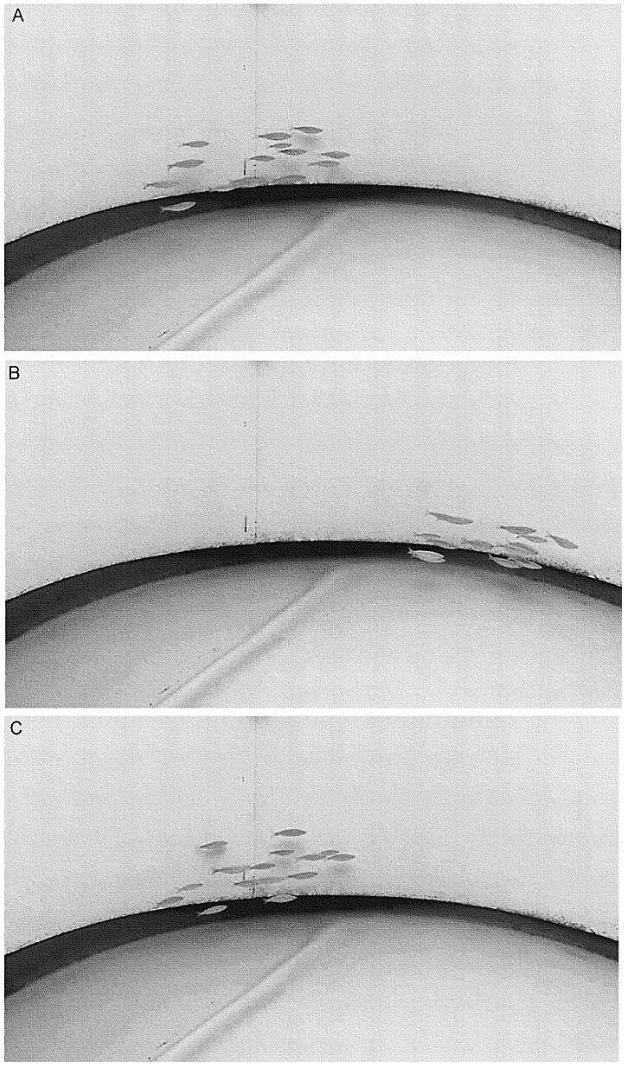

Figure 6 Time-lapse images of n=15 yellow-eyed mullet (*Aldrichetta forsteri*) actively schooling in a 13,000 L tank displaying changes to school morphology (A) immediately before an alarm response from an aerial predator threat, (B) during alarm response, and (C) after one swimming rotation of the tank immediately post alarm response.

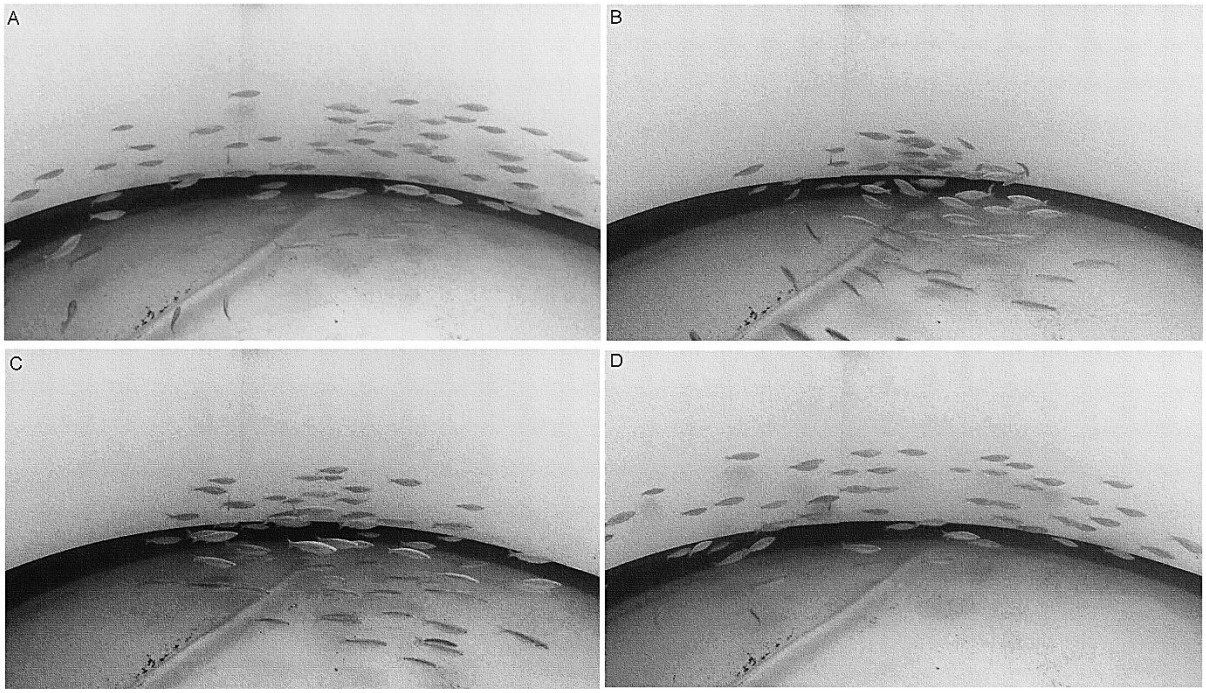

Figure 7 Time-lapse images of n=75 yellow-eyed mullet (*Aldrichetta forsteri*) actively schooling in a 13,000 L tank (A) immediately before an alarm response from an aerial predator threat, (B) during alarm response, (C) immediately post alarm response and (D) after one swimming rotation of the tank post alarm response.

4.5 Discussion

4.5.1 Inter-individual spacing, polarity and swimming velocity

Spacing between individuals in control states was smallest in size group 15 (~1 BL compared with ~1.3 BL in other groups), possibly because of heightened arousal within the group from the small numbers of fish. These results are comparable to mean control 3D NND measurements in minnow (*Phoxinus phoxinus*) (Partridge, 1980) (0.9 BL), saithe (*Pollachius virens*) (0.75 BL), herring (*Clupea harengus*) (0.82 BL) and cod (*Gadus morhua*) (0.64 BL) (Pitcher & Partridge, 1979). Hemelrijk et al. (2010) also found a correlation between increasing NND and group size (10−60) in thick-lipped grey mullet (*Chelon labrosus*) in tank-based studies. There was also decreased NND in all groups in the current study during an alarm response to a simulated aerial predator in comparison to control states (≤1 BL). These findings

are consistent with those of Gerlotto et al. (2006) in schools of anchovy (*Engraulis ringens*), where NND decreased during a predator-initiated alarm response. Greater spacing between yellow-eyed mullet was evident during feeding behaviour when compared with alarm response NNDs in all size groups, with a significant increase seen in size group 150, at around 1.4 BLs. It seems likely that as foraging urgency increased with a dispersed feed item (i.e. pellets), fish preferred more space, to reduce interference by others and increase feeding success.

Maintenance of school structure and rapid propagation of information through fish schools is believed to be strengthened by the close alignment of individuals (Herbert-Read, 2016; Viscido et al., 2007). Therefore, it was expected that polarity in yellow-eyed mullet would be consistent between group sizes under different behavioural states; our results support this theory, with only a 4° range of SA in all instances. This not only suggests that yellow-eyed mullet naturally display high degrees of schooling alignment as part of their behavioural repertoire, but also adds weight to the existing body of knowledge that SA (a local schooling property) is a key component to the success of unified schooling behaviour (Marras et al., 2011). Our results differ from studies reported by Tunstrom et al. (2013) on golden shiner (*Notemigonus crysoleucas*), who found as group size increased (30, 70, 150 and 300 individuals), polarity decreased. However, these differences are probably attributable to changes between schooling behaviour displayed in this study and shoaling behaviour observed in their study.

Swimming velocity was significantly lower in the smallest size group (15) than in all other groups in all behavioural states. Interestingly, the largest size group (150) was significantly slower than size group 75 in response to the aerial predator. Our results are somewhat similar to studies reported on minnow (*P. phoxinus*), which showing decreased swimming velocities when group sizes exceeded 75 individuals (Partridge, 1980). Yet they are different from reports of wild schooling striped mullet (*Mugil cephalus*), which displayed positive correlations between group size and increased swimming speed in their natural environment, where cruising

speeds of 5.6 BL s^{-1} were observed in groups of around 15, and 7.6 BL s^{-1} in groups larger than 50 (Peterson, 1976). Notably, the swimming speeds of this congeneric species were much higher in the wild than were observed in our tank-based studies.

4.5.2 Packing density and school shape

A high degree of spatial isotropy was evident from the evenly spaced distances found in directional mean nearest neighbour data, giving a spherical shape to the free-space surrounding each fish. Comparisons with control states showed decreased spacing between fish during an alarm response and increased spacing during feeding for group size 150 only. This pattern is consistent with findings in saithe (*P. virens*) (Partridge, 1981a) and cod (*G. morhua*) (DeBlois & Rose, 1995). Interestingly, while spacing increased and decreased consistently within each size group, there was considerably more free-space between fish in the two largest size groups under all behavioural conditions. It is assumed that this was due to a higher degree of apprehension in the smallest group of 15 fish, arising from their low numbers in comparison to the hundreds of fish with which they usually school. It is worth noting that the authors considered possible negative effects on spacing that may have been caused by a tank boundary effect discussed in Tunstrom et al. (2013); however, this was ruled out given the low relative densities of fish to tank volume used in this experiment, where the approximate ratio (mass:volume) for each group (15, 75 and 150) was 0.1, 0.5 and 1 kg 1000 L^{-1} respectively.

Pitcher and Partridge (1979) concluded from studies in groups containing <25 individual saithe (*P. virens*), herring (*C. harengus*) and cod (*G. morhua*) that a packing density (free-space) of 1.0 fish BL^3 was a conservative, but realistic, estimate of the volume of area around individuals in fish schools. However, we found that in groups of 15 yellow-eyed mullet the mean volume was ~3.0 BL^3. The volume of free-space increased by 2-3 fold in larger size groups, which contrasts to results reported by Tunstrom et al. (2013), who found that spacing remained stable

with increasing group size in golden shiner (*N. crysoleucas*). However, their methodology calculated packing density by dividing the number of fish in the school by the area (calculated from the span of the school), and multiplying by the mean area of each fish (measured in pixels). This differs from our aggregative method based on 3D measurements of volume (not area) of space around individual fish. Our study suggests that packing density does in fact vary with group size in yellow-eyed mullet, and is not directly influenced by individual fish size, given that all three size groups (15, 75 and 150 individuals) contained fish of near identical mean size.

Fish schools come in all shapes and sizes, and Pavlov and Kasumyan (2000) succinctly reviewed the large variability between fish species. The oblong shape of schools of yellow-eyed mullet in our tank-based studies is similar to school morphology described in findings from both images of offshore wild schools of mullet (*M. cephalus*) (Breder, 1959), and tank-based studies of thick-lipped grey mullet (*C. labrosus*) (Hemelrijk et al., 2010). Observation of school shape in a wild group of yellow-mullet in a shallow estuarine environment highlighted the variability of school morphology, which in this case was evidenced in two different environmental contexts (an artificial tank and a natural estuary). Breder (1959) suggested that the general shape of *Mugil* schools becomes more spherical when fish are not vertically confined within the water column because of depth, which probably explains the differences observed between our wild and tank observations. Saithe (*P. virens*), herring (*C. harengus*), cod (*G. morhua*) and bream (*Abramis brama*) school shapes were described by Pitcher and Partridge (1979) as longest in the x plane (length), followed by y (breadth) then z (height) planes, which matches our findings in yellow-eyed mullet. However, accepting the highly variable nature of school morphology, a general ratio of 3:2:1 (x, y and z) was proposed by Pitcher and Partridge (1979). This differs from our results of 5:2:1, highlighting the variable nature of school morphology between species.

4.5.3 Behavioural response to predator threat

Two distinct predator avoidance behaviours were observed between the small group of 15 fish, and groups of 75 and 150 individuals. The former maintained cohesive school structures, although swimming velocities were lower. When exposed to a simulated predator, schools of 150 individuals were disrupted, with fish at the front of the school doubling back, whereas smaller groups remained cohesive. This appears to be an adaptive behaviour to reduce the volume occupied by the group, and perhaps to reduce predation risk, which was proceeded by a quick return to previous schooling behaviour. A study by Marras et al. (2011) found similar results in herring (*C. harengus*) when, after a startle response, they regained school cohesion after a latency time of ~1 s^{-1}. Litvak (1993) also investigated the effects of aerial predation (kingfisher model) on shoals of golden shiner (*N. crysoleucas*), which displayed an array of behavioural responses including directional change.

4.5.4 Global versus local interaction context

A high degree of individual self-organisation across all size groups was evidenced from adjustments made to inter-individual distances, polarisation and swimming velocity, all of which contributed to overall school cohesion. As previously discussed, NND, SA and velocity are arguably the three key principal factors most often referred to in the literature when describing localised interaction rules between individuals (Herbert-Read, 2016; Hoare et al., 2004; Katz et al., 2011; Parrish et al., 2002; Tien et al., 2004). Alternatively, packing densities could be considered a determinant of overall school size (a global property), and our results support a theory that a combination of both local (individual interaction rules) and global (school size) properties influence the schooling behaviour of yellow-eyed mullet within size groups between 15-150 individuals. However, it should be noted that modelling work by Kunz and Hemelrijk (2012) suggests that beyond 200 fish, global properties are no longer at play in

groups, as individuals are no longer able to perceive all other fish visually and therefore, only local interaction rules can apply with large formations, giving weight to the 'many eyes' hypothesis, which suggests benefits (e.g. reduced predation) increase because greater detection rates are possible with more individuals (Lima, 1995; Roberts, 1996). We therefore suggest that packing density may also be a driver of local interactions in yellow-eyed mullet.

From research in golden shiner (*N. crysoleucas*), Katz et al. (2011) surmised that considering the critical role that individual adjustments to swimming velocity play in maintenance of school structure, it is surprising that very little research effort has been undertaken. The degree of variability in swimming velocity found in our results may give support to the idea that more research is required to elucidate the significance of the role velocity plays in maintenance of school structure. We suggest that modulation of swimming velocity seen in the current study is likely to have been a critical factor in determining interactions between individuals in the maintenance of school structure when group sizes and behavioural states varied. The present experimental design did not enable us to investigate this theory in further detail. Elucidating finer-scale changes in acceleration between individuals in a group and comparing these with the number of changes in NND and SA would be an interesting area for future research.

4.5.5 Estuarine habitat context

Our study findings are of particular relevance to understanding the use of estuary habitat by a mono-species assemblages. Estuarine ecosystems are highly dynamic, with obvious geographical boundaries (i.e. shorelines) (Roy et al., 2001). Combined with daily tidal fluctuations, estuaries (in contrast to offshore waters) possess a finite amount of space in which many species co-inhabit (Roy et al., 2001). Compared with pelagic species, which occupy larger spatial extents, it is likely that a greater number of predator-prey interactions (avian and aquatic) and greater competition for food resources and space exist in confined estuarine

environments. Therefore, these constantly changing dynamics are likely to influence the behaviours and dynamics of schooling fishes strongly.

Studies have shown that success rates at finding food sources are positively correlated with group size, as evidenced in goldfish (*Carassius auratus*) and minnows (*P. phoxinus*) (Pitcher et al., 1982). However, a potentially negative aspect to group living is reduced individual foraging success due to high numbers of individuals competing for the same food resources (Ranta & Kaitala, 1991). Given that food resources are unevenly distributed in estuaries, this may have implications for decision making around migration of individuals between groups (e.g. high group membership might result in less immigration by individuals in other groups). This would be an interesting area for future study. In addition to aiding foraging success, it is also known that the collective behaviours of fish can decrease predatory risks (Cosolo et al., 2010). Successful foraging in great cormorant (*Phalacrocorax carbo*), a natural predator of estuarine species of mullet (Mugilidae) and flounder (*Platichthys flesus*), requires selection of different predatory strategies based on prey ecology (Cosolo et al., 2010). Cosolo and colleagues considered that it was more difficult for cormorant to catch pelagically associated mullet because of their high mobility, collective behaviours and dispersal throughout the water column, in contrast to the more uneven benthic distributions of the flounder.

Our findings throw light upon the impact of group size on interaction rules and decision making (local and global properties) in schools of yellow-eyed mullet during similar behavioural states (predator and foraging) to those experienced by estuarine-inhabiting populations. Yellow-eyed mullet assemblages underwent behavioural changes specific to the group size that they were inhabiting, which were ultimately defined by interaction rules governing the maintenance of cohesion within aggregations. Yellow-eyed mullet showed preference for membership of schools of >15 individuals, and the implications of these larger group sizes are obvious when considering both foraging and anti-predator behaviours necessary for survival in dynamic

estuarine ecosystems. As discussed, larger fish groups benefit from reduced predation risk and greater foraging success (Partridge, 1982). Given that estuaries place greater demands on food resources because of species richness (aquatic and avian), and the resulting increased trophic level and predator-prey interactions, schooling in large numbers is considered a contributing factor to the success of this species. Schooling species such as yellow-eyed mullet are considered to employ a relational set of interaction rules and decisions to maintain their collective behaviours. It must be asked whether these same determinants of group cohesion associate or interact with the foraging success, and/or successful predatory avoidance behaviours, of species in more variable ecological settings. Although presently untested, this notion that collective interaction rules may underlie critical ecological behaviours such as optimal foraging and survival is an endeavour worth pursuing (within the field of ecology).

4.6 Summary

Discussion of decision making and the maintenance of fish school structure is often centred on the role that global (school size) and local (e.g. NND) properties play. We found that group size had a significant impact on the degree of spatial isotropy between aerial predator responses and feeding behavioural states, and that while volume of free-space around individual fish differed depending on group size and behavioural state, distances between fish were directionally evenly spaced and spherical in shape. Our results highlight that school structure cannot be maintained without the co-existence of both global and local properties and therefore, rules which govern school behaviour are intrinsically associated with both school size, and interactions between individuals who are near neighbours. This challenges traditional theories that group structure is maintained by either property, and suggests a wider, more collaborative approach is involved in group living. These interaction rules associated with different behavioural states are likely to influence the wild schooling behaviours of fish species. Results

from the current study may contribute to the development of future research, specifically in large fish aggregates, which will identify the mechanisms used in collective behaviours.

4.7 Ethics statement

All experiments were conducted in accordance with the University of Canterbury Animal Ethics Committee (Ref: 2014/35R). Methods were carried out in accordance with approved guidelines.

5 Phenotypic assortment: effects of group encounter on behaviour and structure in mono-species schools and shoals

5.1 Abstract

Participation in group living is common in around half of all fish species, increasing individual fitness via decreased predation risk and improved foraging success. Group encounters are an integral component of group formation because they provide opportunity for decision-making on whether individuals stay or leave the group; decisions primarily thought to be influenced by body size (phenotype). Assortment opportunities increase with frequency of encounters, and are often higher in enclosed habitats (e.g. estuaries). However, literature relating to phenotypic assortment and its role in the formation and structure of groups has been limited to very few species of schooling or shoaling fish. Our aims were to: (a) identify size-assortive traits associated within previously undescribed mono-species group encounters, (b) quantify interaction effects on group structure, and (c) discuss possible differences in phenotypic assortment between schools and shoals. Using a choice experiment, we investigated sympatric species yellow-eyed mullet (*Aldrichetta forsteri*), an obligate schooling fish, and snapper (*Chrysophrys auratus*), facultative schoolers. Small and large (~2-fold length difference) conspecifics were introduced into a single tank. Three-dimensional stereo-video observations were made under control, aerial predator, diving predator, and feeding behavioural states. Yellow-eyed mullet formed separate size-assorted schools, while snapper formed a single shoal. Changes to group structure varied among species, size-assorted groups, and behavioural states. Our results suggest that swimming behaviour is a key driver of both size-assortiveness and interaction rules governing group formation and structure. This has important implications for understanding the mechanisms that influence group interaction and anti-predator/foraging behaviours in natural fish populations.

5.2 Introduction

Group living is common within the animal kingdom, and given around 50% of all fish species display this behaviour at some stage of their life-cycle (Krause & Ruxton, 2002; Shaw, 1978), the importance of this trait is undisputed. Fish aggregations consist of schools (displaying synchronised and polarised swimming patterns), or shoals (grouped, but unsynchronised swimming patterns), as described in Delcourt and Poncin (2012). Generally, group formation is preferred with individuals possessing phenotypically similar traits, for example body size and species (Hoare et al., 2000; Krause et al., 1996b, 1998; Ranta et al., 1992; Theodorakis, 1989; Ward & Krause, 2001). Benefits include reduced predation risk and increased foraging success (Partridge, 1982). It is suggested that size-assorted behaviour reduces predation risk by reducing individual oddity, and subsequently drawing less attention from predators (Ranta et al., 1994). However, even heterogeneous fish groups, which arguably by their very nature are likely to increase the oddity effect, also display levels of size-assortiveness. This was evidenced in mixed-species shoals of golden shiner (*Notemigonus crysoleucas*) and banded killifish (*Fundulus diaphanous*) (Krause et al., 1996a). It is further suggested, that body size is of greater importance to the determination of group formation than either group size or species composition (Krause & Godin, 1994). Therefore, choosing who you swim with can be considered a fundamental adaptive behaviour evolving from environmental selection pressures. In this study, using body size as a phenotypic trait, we hypothesised that size-assortive behaviour in mono-species group encounters would differ based on species swimming behaviour (i.e. schooling vs shoaling), and that immediate post-predation/foraging responses would also differ as a result.

The immediate responses of schools to external events, such as predation avoidance and foraging activities, differ largely depending on risk type (i.e. avian or aquatic predators) (Fuiman & Magurran, 1994; Templeton & Shriner, 2004), and group composition (Allan,

1986). For example, types of responses to an avian predator strike include changes to school structure (e.g. nearest neighbour distance (NND) and separation angle (SA)) (Vanesyan et al., 2015), depth positioning within the water column (Rieucau et al., 2014b), use of cryptic behaviours (e.g. camouflage, and motionless activity) (Keenleyside, 2012; Leclercq et al., 2010), and changes in swimming velocity, such as the "darting behaviour" observed in guppy (*Poecilia reticulata*) (Vanesyan et al., 2015). Moreover, response strategies (e.g. cryptic behaviour) employed by different species can vary with age, likely driven in part by species specific life-history traits associated with habitat use (Fuiman & Magurran, 1994).

Estuarine ecosystems simultaneously provide important (if not critical) and overlapping habitat for many fish species at various life stages (e.g. ontogenetic change in habitat use between juveniles and adults). This coexistence of many species, and size classes, frequently dictates shared resource use resulting in food competition, as well as exposure to common predators, both avian and aquatic (Crosetti & Blaber, 2016; Elliott & Hemingway, 2008; Koutrakis, 2015). In addition, small geographical home ranges associated with habitat such as estuaries can also lead to increased group interactions among species (Flierl et al., 1999). Frequency of such encounters likely affects individual movement rates (e.g. stay or join another group). All decision-making feeds into top-down/bottom-up feedback systems whereby individual behaviours shape population level spatial distribution patterns, which in turn impact encounter rates at an individual level (Croft et al., 2003). Therefore, changes to group membership are likely to have a direct impact on individual fitness, particularly in populations where group encounters are high.

Species used in the present investigation were chosen for their representation of two naturally occurring sympatric populations, with no published literature on phenotypic assortment for either. Yellow-eyed mullet (*Aldrichetta forsteri*) and snapper (*Chrysophrys auratus*) occupy estuarine and inshore waters of New Zealand and Australia (Morrison et al., 2014; Paulin,

1990; Taylor & Paul, 1998). The former is an abundant, obligate schooling species, while the latter is more commonly seen displaying shoaling behaviour. Both species can be considered estuarine opportunists (i.e. non-resident), and fidelity rates are strongly related to seasonal (e.g. temperature gradients), and/or ontogenetic (juvenile vs adult) near/off-shore migration behaviours (Hartill et al., 2003; Potter & Hyndes, 1994; Potter et al., 2015). Whilst some dietary overlap exists between these species with both readily preying upon small crustacea (Hartill et al., 2003; Potter & Hyndes, 1994), snapper are more typically considered a piscivorous predatory species (Crossland, 1981). Snapper are also likely natural predators of juvenile yellow-eyed mullet since their diet includes small fish of phenotypically similar shape, including pilchards (*Sardinops neopilchardus*) (Godfriaux, 1969). Yellow-eyed mullet and snapper both display different types of swimming behaviours, yet share a similar benthic feeding habitat within estuaries. Snapper are known to occupy small predictable home ranges (hundreds of metres) related to sediment-based food resources in estuaries (Hartill et al., 2003), whereas Mugilidae species (e.g. thin-lipped grey mullet (*Liza ramada*)) are known to travel distances of around six kilometres within an estuary over a 24 h period (Almeida, 1996). Therefore, predation and mono-species foraging behaviours displayed in our tank-based experiments provide synergies for extrapolating possible interactive behaviours in naturally occurring populations of yellow-eyed mullet and snapper.

5.3 Materials and methods

5.3.1. Experimental animals

Tank-based experiments were undertaken at The New Zealand Institute for Plant & Food Research Limited (PFR) Seafood Research Facility in Nelson, New Zealand, using yellow-eyed mullet (*Aldrichetta forsteri*) and snapper (*Chrysophrys auratus*). Yellow-eyed mullet were caught from the Nelson Haven (41.254°S, 173.278°E) in December 2014. Snapper were

randomly sampled from a population of reared fish, spawned from wild sourced brood stock, held at PFR's research facility in Nelson, New Zealand. Fish were held in aerated 5000 L flow through tanks, using filtered seawater pumped from the surrounding Nelson Haven, and water chemistry was maintained at a pH of ~7.6, practical salinity unit (PSU) of 35–36, and dissolved oxygen (DO) at >90%. Mean nominal ambient seawater temperature over the study period (December 2015 – January 2016) was 20.7 °C SD ± 0.3. Stocking densities did not exceed 15–25 kg m^3 and animals were fed a Skretting® pellet diet (Nova ME, Skretting, Australia) twice daily calculated at 2% body mass per day. Mono-species replicates (n=3 replicates totalling 30 fish in each) of yellow-eyed mullet and snapper were formed from 15 small (~100 mm) and 15 large (~250 mm) fish. Mean fork lengths (FL) and mass (± SD) for all 45 fish in each species/size group were: (1) yellow-eyed mullet small: 106 ± 11 mm, 12 ± 5 g and large: 246 ± 7 mm, 206 ± 20 g, (2) snapper small: 116 ± 8 mm, 35 ± 8 g and large: 268 ± 15 mm, 447 ± 71 g. Fish were held in similar sized groups until the experiment was completed. Note that measurements for snapper data only utilised two replicate groups (not three) due to poor quality footage in the third (omitted) replicate. To avoid any possible conditioning effect on results, fish were used only once for experimental exposures.

5.3.2. Experimental tank setup

A 13,000 L (diameter 3.5 m, depth 2.6 m) experimental tank was used to carry out behavioural observations. Seawater was filtered (1 μm) to maximise water clarity. In order to standardise environmental conditions between fish groups, no food was administered 24 h prior to observations and water flow was turned off during observation periods. Animals were lightly anaesthetised (20 ppm, AQUI-S®), transferred from home tanks into the experimental tank and given ~24 h to acclimate, immediately prior to experimental work. Tank apparatus (Fig. 1) consisted of a centrally positioned feeding tube above the tank for administering feed pellets

~15 times at ~30 s intervals. Simulated avian aerial and diving predator models were presented to the fish using a horizontal pulley system positioned ~1 m above the water surface. A synchronised (LED flashing light twice at a 3–5 sec interval at the beginning of recordings) stereo-video camera system (consisting of two Go-Pro® cameras attached to a base plate with a lens separation distance of 420 mm at a 4° inwards incline for each camera), was positioned ~1 m below the water surface and affixed to the tank wall. Standardised start times (between 0730 and 0830 h) were applied to all observations (single group per day) and the observer was obscured from fish view during experiments.

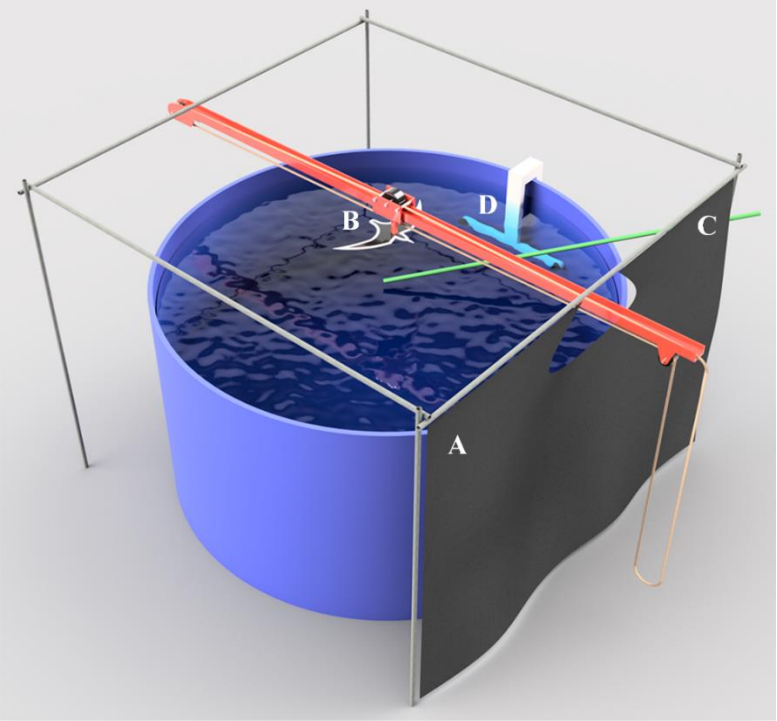

Figure 1 Schematic of experimental tank setup showing (A) shroud to hide observer from fish view, (B) pulley system for simulated alarm response using avian aerial and diving predators, (C) feeding tube and (D) stereo-video camera system. Image credit: Olly Burrow.

5.3.3. Behavioural manipulations

Fish were observed under the following behavioural states: (1) control, (2) aerial predator, (3) diving predator, and (4) feeding. Standardised observations (between groups) were conducted

over a continual 50-min period and video footage divided into the following segments: (a) 0–10 min (acclimation period once camera system placed in tank), (b) 10–20 min (control), (c) 20–30 min (aerial predator), (d) 30–40 min (diving predator), and (e) 40–50 min (feeding). For the aerial predator exposures (c) the avian predator model was pulled across the tank and back ~20 times at ~30 s intervals; the return passage took ~3–5 s. The diving predator exposure (d) was presented by dropping then immediately removing the bird model into and from the tank at ~30 s intervals). Feeding behaviours (e) were investigated using the same commercial pellet feed used for daily rearing (providing continuity) and introduced in ~5 g amounts (mixed pellet size for differences in small and large fish gape) into the tank via the feeding tube. On completion of observations, all fish were transferred back to a holding tank separate to the unused fish before the experimental tank was drained and refilled for transfer of the next group.

5.3.4. Group structure measurements

Variables of nearest neighbour distance (NND), separation angle (SA), and swimming velocity, commonly used to quantify school structure (Masuda & Tsukamoto, 1998; Santana-Garcon et al., 2014)), were analysed during each of the four behavioural states. Measurements of NND and SA were confined to fish displaying schooling behaviours. Therefore, only the behavioural responses of yellow-mullet could be quantified as snapper did not show schooling behaviours throughout experimentation. Velocity and shoal area were the only group behaviours measured in snapper. Measurements were made from stereo-video footage using SeaGIS EventMeasure software (SeaGIS, 2017). Briefly, the software measured NND from the 3D mid-point of each fish (3D mid-point is calculated from the mean of 3D head and tail positions (FL)), SA from the angle between the directions of each pair of fish (fish direction defined by the 3D head and tail coordinates), and swimming velocity from 3D head positions measured at different time points (i.e. t1 & t2). Only fish clearly visible (head and tail) in each frame were selected for measurement. The NND and SA were computed for each individual, then a mean was

calculated using all individual measures made over n=3 replicate frames (~30 s apart). During aerial and diving predator, and feeding behavioural states, fish were measured ~5 s after the stimulus event to standardise all measurement conditions among fish groups. Due to poor visibility, snapper swimming velocity analysis was only possible from n=2 (not three) replicate groups. Shoal size (area m^2) was calculated from a 2D irregular polygon using x and y coordinates from vertices around the periphery of the group (three measurements from three separate frames in each replicate group (n=6)) applied to the following formula:

$$\text{Area} = \frac{\sum_1^{n-1}(x_i y_i - x_{i+1} y_{i+1}) + (x_n y_n - x_1 y_1)}{2}$$

Where xi is the x coordinate of vertex i and y is the y coordinate of the i^{th} with the final term repeating the first to finish at the starting vertex. Time spent in lower, middle and upper depth areas (each defined as a third of the water column) by yellow-eyed mullet were measured by calculating percentage time spent in each area from 10 repeat measures (separate frames) from each of the three replicate groups (i.e. n=30 measurements for each position).

5.3.5. Statistical analysis

Data for NND, SA and swimming velocity were analysed using R v.3.3.2. A linear mixed model (LMM) was fitted with restricted likelihood to account for the unbalanced nature of the data. Response variables failing assumptions were log-transformed. Behaviour, fish size and their interaction were fitted as fixed effects and replicate group as a random effect. Error bars are means ± 95% CI unless otherwise stated. Spatial area analysis for snapper was carried out in SigmaPlot v.12.5 using 1-way ANOVA to compare the behavioural states means, and data log-transformed after failing equal variance assumption. Pairwise comparisons were tested using Tukey test and significant was accepted at $P \leq 0.05$.

5.4 Results

5.4.1 Qualitative analysis of assortiveness behaviours and immediate alarm/foraging responses

Qualitative observations showed that after initial introduction, small and large yellow-eyed mullet groups formed and maintained separate size-assorted schools, with the smaller fish persistently occupying a higher depth position. This behaviour was consistent during all behavioural manipulation states. Small yellow-eyed mullet in control states displayed frequent darting (defined as increased/decreased swimming velocity), and directional change in swimming behaviour, whereas large fish swam at a seemingly consistent rate in a clockwise direction in the lower half of the water column. In contrast, snapper formed and maintained a single shoal and displayed no apparent size-assortiveness throughout all behavioural states. Control fish occupied the full tank area in the lower half of the water column.

Immediate behavioural responses by small yellow-eyed mullet to predation risks included increased frequency of darting behaviour during both aerial and diving predator states. Large yellow-eyed mullet behaviours included benthic positioning, reduction in velocity and school area, and occasional loss of group cohesion (unpolarised due to double-back effect) during the aerial predator behavioural state. Further reductions in swimming velocity (bordering on motionless activity), and packing density were present during aerial predator threat response in large yellow-eyed mullet. Foraging resulted in increased separation distances between small and large yellow-eyed mullet groups, and an immediate loss of polarity, with a 'ball' affect around the food source. Also, an apparent feeding hierarchy developed, with small yellow-eyed mullet feeding separately to large, appearing to be competitively excluded.

The immediate responses of snapper to the aerial predator also included reduced shoal area, slower swimming velocity, adoption of a more benthic depth position, and some individuals adopted cryptic behaviours (including motionless activity and camouflage tactics (black

striping)). These responses heightened during the diving predator state. Of particular interest, two snapper left the safety of the group to undertake predator inspection. Packing density increased during snapper foraging, with no apparent feeding hierarchy within the shoal.

5.4.2 Group structure measurements

5.4.2.1. Nearest neighbour distance

When using absolute length (mm) to compare differences in mean NND between small and large yellow-eyed mullet (Fig. 2A), the small fish group displayed significantly less interindividual spacing in all behavioural states ($P < 0.001$). However, the same comparisons using BL (Fig. 2B), as a unit of measure for mean NND, found increased spacing (~15%), in the small fish group only, compared with large fish during their alarm response to a simulated aerial predator ($P = 0.005$) (Fig. 2B). While both measurements are given in figures, the BL method of comparison is deemed more relevant and therefore, statistical analyses are interpreted accordingly. All means are tabulated.

Mean NND in both small and large yellow-eyed mullet, in all behavioural states, ranged between 0.7 and 0.9 BLs, and controls were similar with mean separation distances of 0.77 and 0.79 BLs respectively (Fig. 2B, Table 1). Within size group NND comparisons of large and small yellow-eyed mullet found no significant difference in the control group when compared to all three treatment groups (linear mixed model (LMM; Wald test statistic = 49.455, $DF = 3$, $P > 0.05$ for all). Comparisons between groups also found no significant difference in corresponding control or treatment groups between large and small fish (LMM; Wald test statistic = 9.328, $DF = 3$, $P > 0.05$ for all). There was no significant interactive effect between fish size and behavioural state (LMM; Wald test statistic = 7.186, $DF = 3$, $P = 0.066$).

Table 1 Measurements of small and large yellow-eyed mullet (*Aldrichetta forsteri*) and snapper (*Chrysophrys auratus*) nearest neighbour distance (NND), separation angle (SA) and swimming velocity during control, aerial predator, diving predator and feeding behavioural states. Data represented as means ± 95% CIs.

Measurement variable	Species	Fish size	Behavioural state			
			Control	Aerial predator	Diving predator	Feeding
NND (BL)	Yellow-eyed mullet	Small	0.77 ± 0.05	0.86 ± 0.05	0.69 ± 0.03	0.94 ± 0.09
		Large	0.79 ± 0.04	0.75 ± 0.05	0.70 ± 0.05	0.87 ± 0.05
SA (°)	Yellow-eyed mullet	Small	17.97 ± 2.29	22.38 ± 2.96	19.49 ± 2.68	22.19 ± 2.69
		Large	13.68 ± 2.03	15.64 ± 2.23	19.42 ± 2.80	17.19 ± 2.54
Velocity (BL $^{s-1}$)	Yellow-eyed mullet	Small	3.13 ± 0.32	2.61 ± 0.18	1.74 ± 0.19	4.18 ± 0.27
		Large	3.29 ± 0.11	3.24 ± 0.14	2.54 ± 0.18	3.62 ± 0.13
	Snapper	Small	3.82 ± 0.26	2.08 ± 0.40	1.85 ± 0.29	2.71 ± 0.85
		Large	1.15 ± 0.09	0.88 ± 0.19	1.18 ± 0.28	1.16 ± 0.27

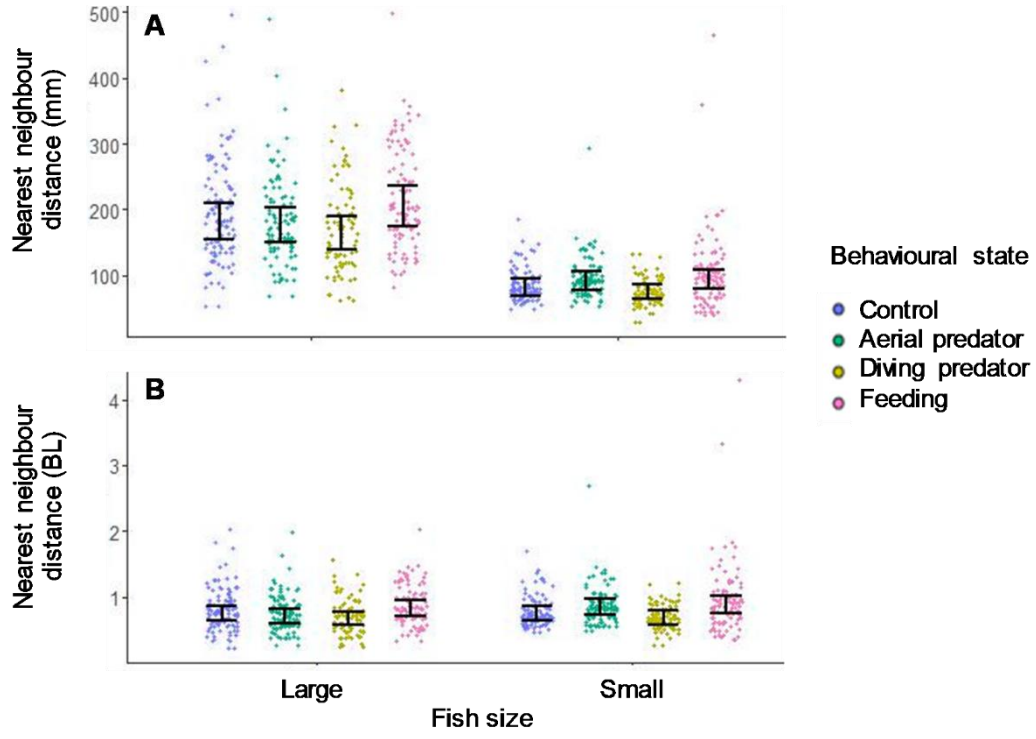

Figure 2 Nearest neighbour distances (A: mm, B: body length (BL (fork length)) yellow-eyed mullet (*Aldrichetta forsteri*) during control, aerial predator, diving predator and feeding behavioural states. Dots represent individual fish and error bars are 95% CI. If no overlap exists the difference between groups is considered significant with significance accepted at $P \leq 0.05$.

5.4.2.2. Polarity

Mean SAs ranged between ~14° and 22° for both small and large yellow-eyed mullet, in all behavioural states (Fig. 3, Table 1). Within size group comparisons of yellow-eyed mullet control vs all three treatment groups in both small and large fish showed no significant difference in SAs (LMM; Wald test statistic = 12.406, *DF* = 3, *P* > 0.05 for all). Between size group comparisons showed a significant decrease in SA during the aerial predator response in large fish compared to small (LMM; Wald test statistic = 24.555, *DF* = 1, *P* = 0.044). There was no significant interactive effect between fish size and behavioural state outcomes in yellow-eyed mullet (LMM; Wald test statistic = 3.257, *DF* = 3, *P* = 0.353).

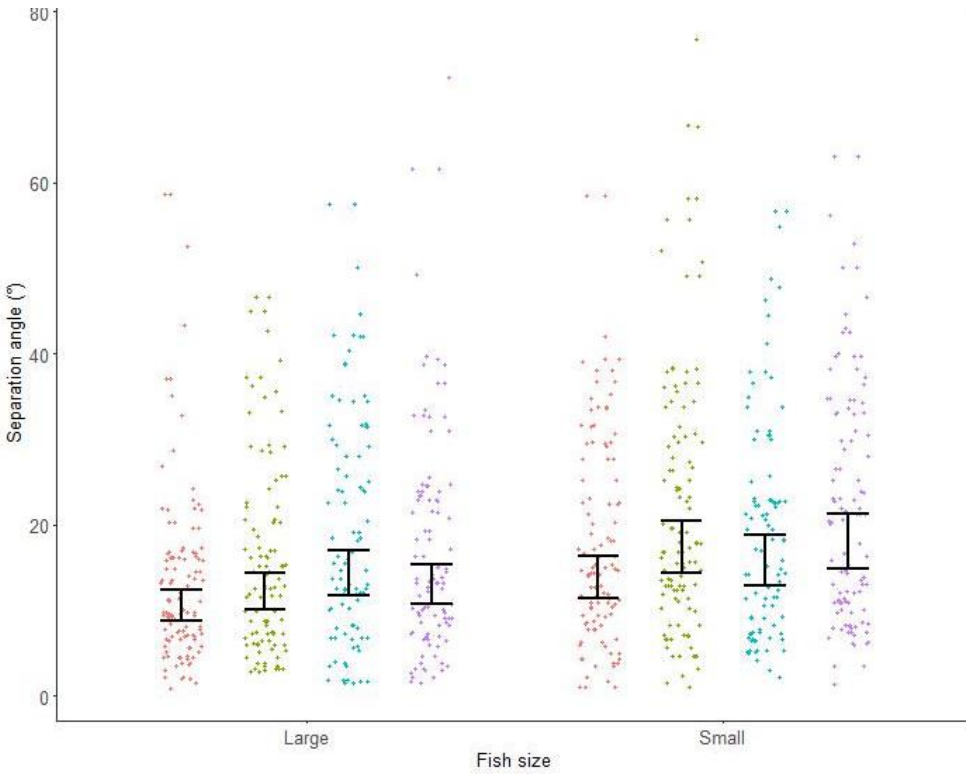

Figure 3 Mean separation angles between large and small groups of yellow-eyed mullet (*Aldrichetta forsteri*) during control, aerial predator, diving predator and feeding behavioural states. Dots represent individual fish and error bars are 95% CI. If no overlap exists the difference between groups is considered significant, with significance accepted at $P \leq 0.05$.

5.4.2.3. Velocity

Mean velocity in small and large yellow-eyed mullet ranged between 1.74 and 4.18 BL $^{s-1}$ during all behavioural states (Fig. 4B, Table 1). In control groups for both size groups, fish swam at similar velocities of around 3.1 and 3.3 BL $^{s-1}$. Within size group comparisons showed swimming velocity was significantly reduced in the small yellow-eyed mullet control group compared with feeding (LMM; Wald test statistic = 521.231, *DF* = 3, *P* = 0.045). Small fish also swam significantly slower during the diving predator state compared with aerial predator or feeding behavioural states (LMM; Wald test statistic = 521.231, *DF* = 3, *P* = 0.021 and P = 0.0144 respectively). There was a significant interactive effect between fish size and behavioural state (LMM; Wald test statistic = 78.354, *DF* = 3, *P* < 0.001).

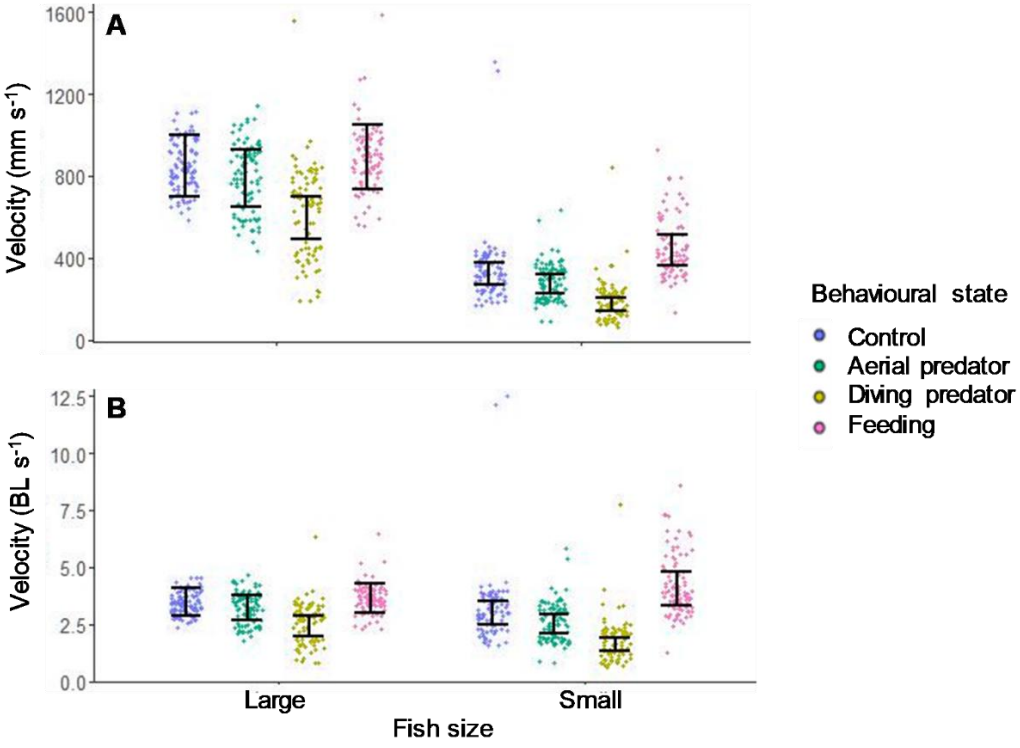

Figure 4 Swimming velocity (A: mm s^{-1}, B: body length (BL, fork length) of yellow-eyed mullet (*Aldrichetta forsteri*) during control, aerial predator, diving predator and feeding behavioural states. Points represent individual fish and error bars are 95% CI. If no overlap exists the difference between groups is considered significant with significance accepted at $P \leq 0.05$.

Mean swimming velocity in small and large snapper ranged between ~1 and 4 BL s^{-1} during all behavioural states (Fig. 5B, Table 1). Comparison of mean swimming velocities within each snapper size group showed that large fish swam significantly faster in the control compared with the aerial predator group (LMM; Wald test statistic = 66.495, *DF* = 3, *P* = 0.031). In the small size group, control fish swam significantly faster than all three behavioural states by at least 2-fold (LMM; Wald test statistic = 66.495, *DF* = 3, $P < 0.05$ for all). Between group comparisons showed that large snapper swimming velocity was significantly reduced in all groups compared to small fish (LMM; Wald test statistic = 283.265, *DF* = 1, $P < 0.05$ for all). There was a significant interactive effect between fish size and behavioural state (LMM; Wald test statistic = 9.533, *DF* = 3, *P* = 0.022).

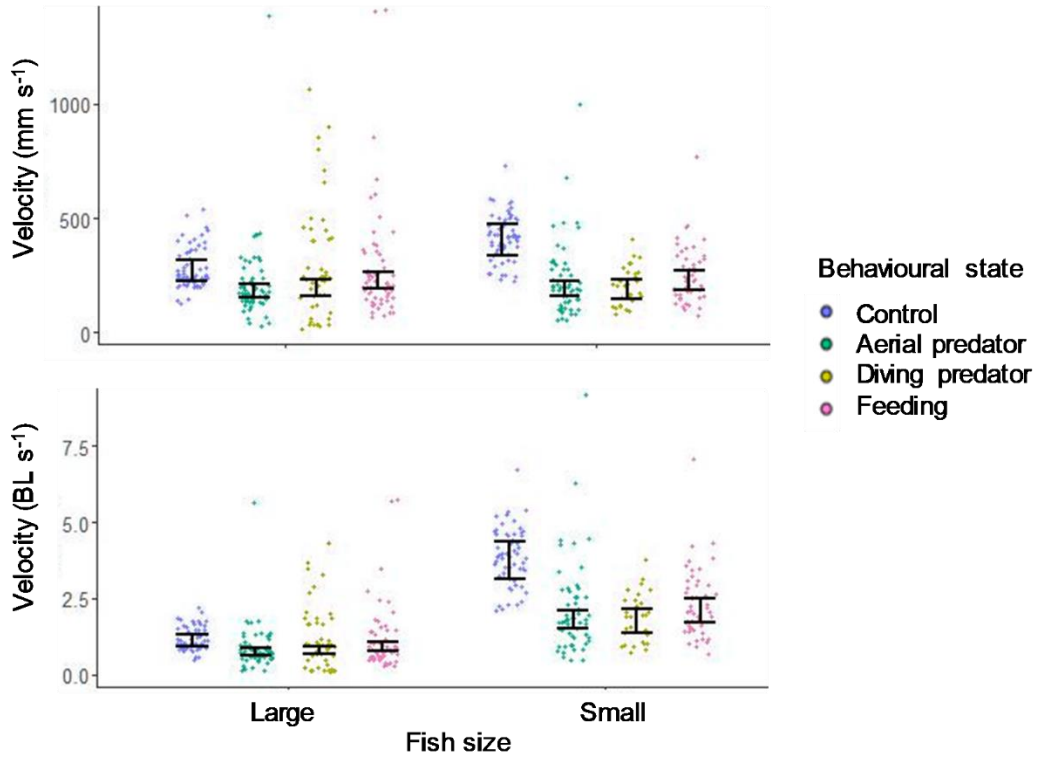

Figure 5 Swimming velocity (A: mm s^{-1}, B: body lengths (BL, fork length) of snapper (*Chrysophrys auratus*) during control, aerial predator, diving predator and feeding behavioural states. Points represent individual fish and error bars are 95% CI. If no overlap exists the difference between groups is considered significant with significance accepted at $P \leq 0.05$.

5.4.2.4. Depth positioning of yellow-eyed mullet schools

Small yellow-eyed mullet were continuously positioned above the large fish in all behavioural states. Small and large yellow-eyed mullet control fish spent >90% of their time in the upper and middle tank depth regions respectively (Fig. 6). During the aerial predator threat, both sizes spent equal amounts of time in the middle position; however, during the diving predator response, whilst larger fish remained in middle region, small fish split their time 40/60 between upper and the middle regions. During foraging, large fish spent >60% of the time in the lower region while small fish were in the upper region >50% of the time.

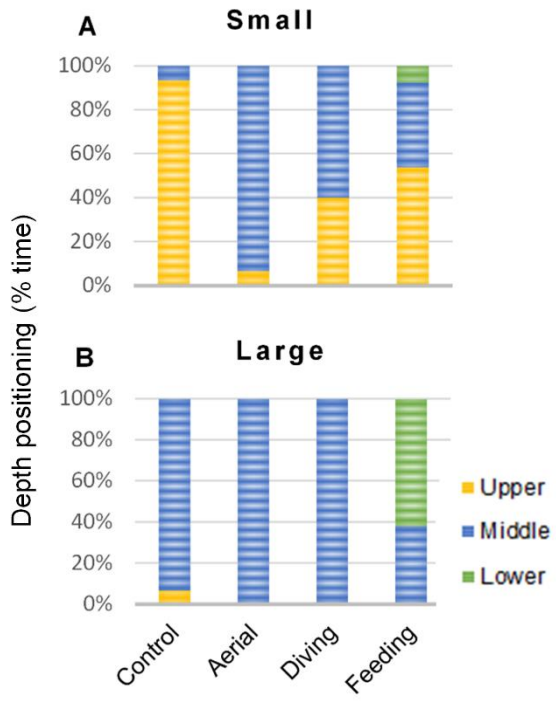

Figure 6 Percentage time spent by small (A) and large (B) yellow-eyed mullet (*Aldrichetta forsteri*) in lower, middle and upper depth positions of the water column during control and post-stimuli aerial predator, diving predator and feeding behavioural states.

5.4.2.5. Snapper shoal area

The mean 2D spatial area occupied by snapper ranged between ~0.2 m^2 and 1.1 m^2 across all four treatment groups (Fig. 7). Control fish occupied significantly more area than aerial

predator, diving predator or feeding behavioural states, by between 2 and 4-fold ($P < 0.001$ for all). Although areas were similar between aerial and diving predator fish ($P = 0.051$), during feeding, fish spacing increased significantly compared with both aerial and diving predator states ($P < 0.001$ for both).

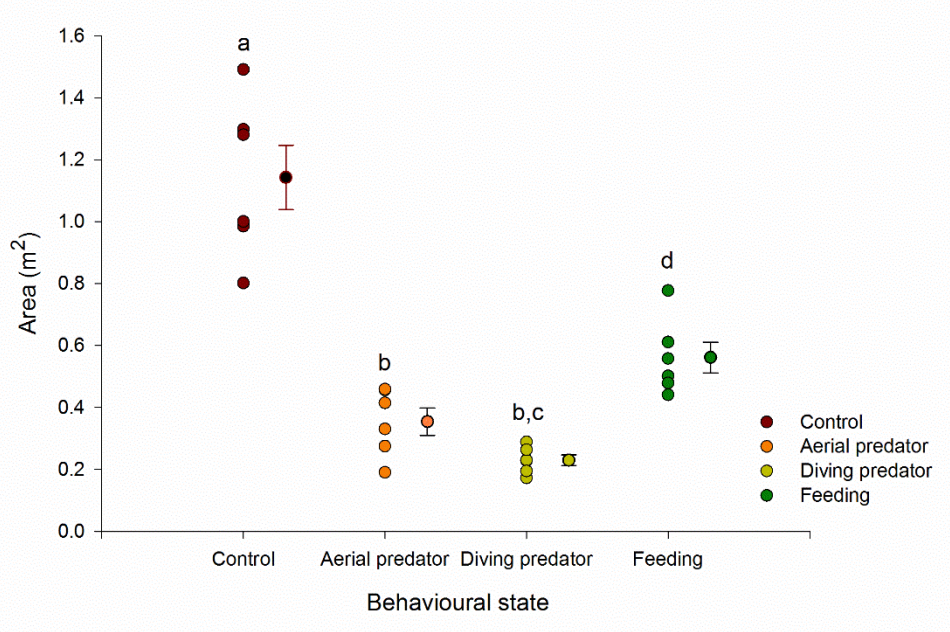

Figure 7 Two-dimensional spatial area occupied by snapper (*Chrysophrys auratus*) during control, aerial predator, diving predator and feeding behavioural states. Points represent raw data, while points with error bars represent means ± SE. Significant differences are indicated with differing letters and significance accepted at $P \leq 0.05$.

5.5 Discussion

Decision-making on group membership is an essential element of fish aggregation (Croft et al., 2003). Little is known about the mechanisms underlying such encounters; for example, phenotypic assortment, nor their effect on group structure (Croft et al., 2003). To address this shortfall we used a choice experiment to elucidate the interactions and dynamics associated with group formation and structure in mono-species assemblages of small and large yellow-eyed mullet (*Aldrichetta forsteri*), and snapper (*Chrysophrys auratus*); species with overlapping habitat use.

5.5.1 Assortiveness and immediate response behaviours

Group composition is thought to play a key role in formation decisions made by individuals upon encountering other groups (Croft et al., 2003; Jones et al., 2010). The importance of affiliation with phenotypically similar individuals is evidenced in size-assortive formations in several species. For example, European minnow (*Phoxinus phoxinus*) (Ward & Krause, 2001), golden shiner (*Notemigonus crysoleucas*) and banded killifish shoals (*Fundulus diaphanous*) (Krause & Godin, 1994; Krause et al., 1996a; Krause et al., 1998), and three-spined sticklebacks (*Gasterosteus aculeatus*) (Ranta & Lindstrom, 1990; Ranta et al., 1992) all form schools with similar sized individuals, rather than schools with a wide range of sizes. However, this is not a 'rule' applied by all, and variation in phenotypic assortment exists between species, and motivational states (Rodgers et al., 2014). Our results support this finding with yellow-eyed mullet displaying assortiveness behaviour (forming separate size-based sub-groups), whereas snapper formed a single non size-assorted shoal. This suggests that phenotypic selectiveness is less important in fish displaying shoaling behaviour. Giving weight to this theory, Ranta and Lindstrom (1990) also found that schools of small and large three-spined stickleback (*G. aculeatus*), when combined in the same tank, separated into two size-based schools. Also, Krause (1994) found that mixed-size shoals (as opposed to schools) of chub (*Leuciscus cephalus*) did not display size-assortiveness, unless their motivational state changed (i.e. predation threat). Differences in size-dependent swimming behaviour were also seen in yellow-eyed mullet. Large fish displayed continuous clockwise schooling under all behavioural states (excluding immediate predator and feeding responses), compared with darting behaviour (constant change in direction) in small fish. These size-dependent differences in schooling responses perhaps indicate a level of phenotypic plasticity that may also influence assortment traits. It is reasonable to assume that differences in swimming behaviour (i.e. schooling vs

shoaling), both between and within species, influence decision making on phenotypic assortment.

Immediate behavioural responses resulting from foraging and predator avoidance activities vary between fish species (Fuiman & Magurran, 1994; Lukoschek & McCormick, 2002). These can include changes to school structure (e.g. swimming velocity, NND and SA) (Vanesyan et al., 2015), depth positioning (Rieucau et al., 2014b), and the use of cryptic behaviours, such as change in skin colouration (background camouflage adaptation) and motionless activity (Keenleyside, 2012; Leclercq et al., 2010). Yellow-eyed mullet maintained size-assorted groups during foraging, and given the differences in body size, and apparent competitive exclusion (not measured) exhibited by large fish upon small, it is not surprising that feeding dominance was apparent in larger fish. A competitive foraging advantage was also found in larger chub (*L. cephalus*) (Krause, 1994). However, no such size-assortment dynamics and associated foraging hierarchy were displayed in snapper, as snapper of both size classes appeared to forage with equal success. A key advantage in group formation is increased foraging success (Partridge, 1982). However, in doing so it also creates interindividual competition as group size increases, and therefore negatively impacts individual fitness (Partridge, 1982). It is likely that yellow-eyed mullet maintained distinct size-based schools for this reason. Perhaps, though, as seen in snapper, adherence by individuals to group interaction rules in shoals doesn't result in reduced foraging success among members in small group sizes. Given there were only 20 snapper in mixed-size shoals, whilst not measured directly in this study, it is unlikely these groups consisting of few individuals would have resulted in interspecific competition.

The immediate alarm response strategies demonstrated by yellow-eyed mullet and snapper, after the simulated predator models (aerial and diving), were considerably different. Whist both species displayed group structural changes (i.e. depth positioning, NND, SA, swimming

velocity), snapper additionally engaged in cryptic behaviours (i.e. black striping of the skin and motionless activity) with greater frequency during the driving predator threat. This type of anti-predator behaviour is common in many aquatic species that utilise a benthic habitat at some stage of their life-cycle (Keenleyside, 2012). An example of this is motionless activity in guppy exposed to an aerial predator (*P. reticulata*) (Templeton & Shriner, 2004). Whilst snapper also utilised both of these strategies, to the human eye, camouflage (black stripes) was noticeably ineffective against a background olive green experimental tank, and highlights the limitations of the behaviour, given snapper are not chameleon-like in their camouflage abilities. Therefore, the success of cryptic colouration, as an anti-predator defence, relies entirely on the blending of skin pigmentation into the immediate background, which is known to have an ontogenetic component given pigmentation increases with growth (Fuiman & Magurran, 1994). The behavioural differences found between yellow-eyed mullet and snapper highlight species-specific strategies (particularly predator avoidance) employed by fish that, having been exposed to different selection pressures, have evolved very different swimming behaviours (i.e. shoaling vs schooling).

Phenotypic oddity is thought to reduce individual fitness by increasing the likelihood of individuals standing out to predators and, therefore, is a key driver of phenotypic homogeneity in group formation (Ranta et al., 1994; Theodorakis, 1989), for example silvery minnows (*Hybognathus nuchalis*) (Landeau & Terborgh, 1986). This was certainly true of yellow-eyed mullet in the current study; however, it was not true of the mixed-sized shoal of snapper. Applying the oddity theory then raises interesting questions around who joined whom. Was it consensual, or did one snapper size group leverage off the other? Studies in three-spined sticklebacks (*G. aculeatus*) showed higher predation rates in larger fish associated with mixed-size groups (Kulling & Milinski, 1992). Therefore, when considering body size effects, there is no advantage to large fish having small fish join the group, because it will make them stand

out, increasing the oddity effect. If it was the small snapper that joined the large, perhaps they saw the large fish as a refuge (safety in numbers), but this would likely disadvantage the large fish, based on the above theory. Results from studies on guppy (*P. reticulata*) showed shoal choice was strongly size-assortive in large fish, but not in small fish (Jones et al., 2010). Combined with our own results, this suggests that it was the small snapper which joined the large ones. This would be another interesting area of further research to identify migration patterns of individual fish (e.g. stay/join) related to size-assortiveness in group formation under differing motivational states.

Reciprocal altruism is a contentious area of ethology involving cooperation between individuals (Silk, 2013), and is suggested to play a role in the evolutionary stability of group composition (Croft et al., 2003; Pitcher, 1991). Instances of altruism were evidenced in stickleback (*G. aculeatus*) partnership behaviours (Milinski & Kettler, 1990). Whilst not included in the experimental design of the current study, interestingly, we observed two instances of possible altruistic behaviours in snapper groups, namely herding of a separated individual, and predator inspection. A single occurrence was noted of a large snapper shepherding a small fish (motionless and displaying camouflage behaviour), back into the group, after having separated from other individuals. Because snapper used in these experiments were reared in captivity from the same broodstock, it is highly likely that these two individuals were genetically related; therefore, did the larger fish display a type of 'paternal care' (the 'selfish gene hypothesis' (Coyne & Sohn, 1978; Dawkins, 1976)), or alternatively, was it a case of mistaken identity (Croft et al., 2003). Perhaps, it was a selfless act to help a younger conspecific displaying obvious signs of stress, but the more likely scenario is that it was an act of reciprocal altruism (Silk, 2013), the motivation of which would ultimately be to decrease the helper fish's predation risk, by increasing group numbers. In addition to the observed individualist behaviours, a second instance was observed, where two snapper

departed from the shoal and engaged in predator inspection behaviours. Predator inspection is considered an altruistic behaviour and an important component of risk assessment (i.e. level of threat) (Milinski, 1992; Pitcher, 1991). We suggest that given snapper engaged in this behaviour (single observation) within the current study, that it increases the likelihood of the presence of altruistic behaviour in this species. Certainly, these limited observations are not enough to support this theory, or to elucidate possible reasons for its existence. However, these types of questions are gaining increasing research attention among fish ethologists (Balcombe, 2016), and sentiency would be an interesting topic for future investigation in group behaviour.

5.5.2 School structure: NND, SA, depth positioning and swimming velocity

5.5.2.1. Aerial and diving predator avoidance strategies

Predation risk modifies group structure, making schools more aligned and compact, and also results in changes to swimming velocity (Bode et al., 2010). In response to predation risk, group formation is also often preferred with phenotypically similar conspecifics, as seen in banded killifish (*F. diaphanous*) when presented with an aerial predator threat (Krause & Godin, 1994). This was also evidenced by results from the present study with yellow-eyed mullet maintaining size-assorted schools in response to predation threats. However, both small and large yellow-eyed mullet anti-predator strategies resulted in changes to group structure, and while similar trends were shown between size groups, the level of response differed.

Interestingly, mean NND in both small and large yellow-eyed mullet was maintained at a constant level in control and treatment groups. This indicates that yellow-eyed mullet quickly resumed an optimum nearest neighbour distance post disturbance. A strategy that is likely very important for the successful maintenance of group structure. Although, smaller fish adopted a deeper and more centralised depth position in the water column, post predation threat, (not measured). Perhaps indicating movement to a position of increased safety closer to, but

remaining separate from, larger fish. It is more likely a consequence of collision avoidance with larger fish via maintenance of a higher depth position. This type of anti-predatory behaviour is not uncommon in fish species (Domenici et al., 2007). Decreased depth positioning was also observed in herring (*Clupea harengus*) exposed to predation threat (Pitcher et al., 1996). When compared with post-feeding behaviour, NND in both aerial and diving responses were reduced. Similarly, Tien et al. (2004) found reduced NND in shoals of creek chub (*Semotilus atromaculatus*), and blacknose dace (*Rhinichthys atratulus*), during a simulated predator threat.

Changes to school structure during predation also included measurement of group polarity. Both small and large yellow-eyed mullet displayed similar mean SA between individuals in control, and treatment groups. This suggests that a level of ontogenetic cognitive plasticity (growth related) is not involved in optimisation and maintenance of group structure. Therefore, schooling behaviour may be fully developed in juveniles of this species. To the authors' knowledge there are no other studies that have investigated this phenomenon in different life-stages of yellow-eyed mullet, and this would be an interesting area for further research involving longitudinal studies.

As previously mentioned, reduction in swimming velocity is also a common response strategy to predation risk, and is likely related to levels of apprehension. Compared to control fish, mean swimming velocity was slower in small yellow-eyed mullet in response to an aerial predation threat. Both small and large fish showed similar reductions in swimming velocity during aerial and diving predator threats. This may also give support to our theory of non-ontogenetic related behavioural development.

Unlike yellow-eyed mullet, in snapper we saw significant differences in velocity between small and large fish and the large fish displayed a slower swimming velocity in all behavioural states.

This was most apparent in control conditions, where small fish demonstrated ~3-fold faster swimming velocities than larger fish. Although shoals displayed motionless activity during immediate behavioural response to a predator, perhaps the instantaneous and fleeting darting tactics utilised by small snapper after an avoidance response (which were of too shorter duration to analyse), indicated a preference to move into perceived safety zones associated with larger fish, rather than to resume routine swimming behaviours consistent with large fish.

5.5.2.2. Feeding behaviour

Foraging behaviours arise from associations between individuals within the same groups, and/or from group encounters (intra- or interspecific). Whilst foraging interactions are an important mechanistic consideration for group formation, they are poorly described in fish species (Lukoschek & McCormick, 2002). Selection pressures, resulting from competition for food resources, may have led to the rise of size-assorted group formation by reducing intra-specific competition between individuals within cohorts (Krause et al., 1996b). This theory is supported in the current study, with small yellow-eyed mullet spending the majority of time foraging in the top third of the water column, whilst large fish conversely foraged more frequently in the lower third. Ranta and Lindstrom (1990) discussed cost-benefit analysis associated with school formation, and suggested that as group size increases, benefits decrease (including foraging success). It is likely that competition contributed to small yellow-eyed mullet not joining the larger fish during foraging and instead choosing to forage as a sub-group. The consequence of size-assortment in natural populations, especially within geographically confined habitats, is increased group density, which in turn increases inter-group interaction rates and, therefore, foraging pressure. Croft et al. (2003) suggested that some groups may then seek low group density areas. This could potentially impact their foraging success given uneven food distribution patterns in estuaries, and the dynamic and rhythmic ecological changes occurring in these ecosystems. However, increased group encounter rates within spatially

restricted habitats may improve individual fitness due to increased opportunity to join groups of a more similar size as the fish grow, as previously suggested by Croft et al. (2003).

Foraging resulted in similar behavioural changes to group structure in small and large fish, during control and treatment groups. Foraging strategy appeared to be comparable for individuals of different sizes (within a species), and rather appears to vary in response to intraspecific competition, with fish adopting spatial behaviours that best advantage individual foraging success. Similarly, this trend in NND continued with SA in both size groups after a feeding event compared with control groups. It should be considered, therefore, that both sets of results suggest predation threat, and foraging behaviour have an equally disruptive effect on school structure regardless of fish size. This gives weight to our theory that the development of schooling behaviour happens early on in the life-cycle of natural populations..

Swimming velocity decreased by >3-fold during foraging in wild cod schools when exposed to abundant food resources (*Gadus morhua*) (DeBlois & Rose, 1995), which differs from our findings in yellow-eyed mullet. This is possibly related to wild vs tank-based studies respectively. However, it is a reasonable assumption, given yellow-eyed mullet initially abandoned cohesive schooling behaviour to feed then returned to previous collective behaviours shortly after the feeding event, that the resumption and maintenance of swimming velocity after a feeding event conferred an advantage. Conversely, in snapper, we found that swimming velocity did not return to, nor exceed, rates found in control fish of either size, and this was likely due to a lack of intraspecific competition within the shoal.

5.6 Summary

Group encounters are a key driver of both phenotypic assortment and interaction rules governing group formation and structure. Estuarine habitat is utilised by fish species commonly displaying schooling and/or shoaling behaviours. Collective behaviours related to sympatric

schooling and shoaling fish, have important implications for understanding the mechanisms that influence group interaction, and structure, resulting from anti-predator and foraging behaviours in natural fish populations. Our findings suggest that heterogenic species successfully coexist in overlapping habitat, partly because they have each evolved different behavioural adaptions allowing them to optimise individual anti-predator and foraging strategies. Yellow-eyed mullet, an obligate schooling species, show distinct size-assortive group formation behaviour under experimental conditions. Whereas snapper, displaying shoaling behaviour, did not show the same preferences for size-assortment of their aggregations. In line with these results, we propose that a high degree of size-assortiveness is likely found in natural populations of yellow-eyed mullet, which would increase group density within these spatially limited habitats. The existence of numerous, size-assorted schools of yellow-eyed mullet within confined habitat may then increase the frequency of group encounters, and arguably predator-prey interactions, whilst also increasing foraging competition between groups. Understanding selection pressures that drive phenotypic assortment in group formation may provide important insight in understanding how decision-making impacts individual fitness in fish groups.

5.7 Ethics statement

All experiments were conducted in accordance with the University of Canterbury Animal Ethics Committee (Ref: 2014/35R). Methods were carried out in accordance with approved guidelines.

6 Lateralisation of visual function in yellow-eyed mullet (*Aldrichetta forsteri*) and its role in schooling behaviour

6.1. Abstract

Lateralisation of cognitive function is a common phenomenon found throughout the animal kingdom. Strong biases in functional behaviours exist because of asymmetrical brain hemispheres which differ in structure and/or cognitive function. In fish, lateralisation is involved in visually mediated behaviours such as schooling, predator avoidance, and foraging, and is considered to have a direct impact on fitness. The yellow-eyed mullet (*Aldrichetta forsteri*), is an estuarine and coastal species found commonly throughout temperate regions of Australia and New Zealand. This study sought to quantify visual laterality in yellow-eyed mullet, to determine visual asymmetries and the significance of lateralisation, and the factors which influence functional behaviours in schooling fish. Our approach to study design was to conduct a series of tank-based experiments investigating; a) individual and population level lateralisation, b) schooling behaviour, and c) optic lobe anatomy. Yellow-eyed mullet showed individual variation in direction and strength of lateralisation in juveniles, but significant population level bias in adults. Right eye biased fish had a larger right optic lobe than control fish. Strongly lateralised fish showed a tendency to occupy positions of safety within the school more frequently than control fish. In combination with observed differences in schooling behaviour, the possibility of ontogenetic plasticity in behavioural lateralisation and optic lobe morphology is suggested. These findings highlight the need for research into the genetic and environmental factors (epigenetics) which drive functional behaviours such as schooling, feeding and aggression.

6.2. Introduction

Differences in structure or function of left and right brain hemispheres have given rise to bias in behaviours (e.g. side biases) throughout the animal kingdom (Bisazza et al., 1998; Malashichev & Deckel, 2006; Vallortigara et al., 2011). This phenomenon is referred to as lateralisation and is thought to have evolved due to selective advantages associated with hemisphere specialisation (e.g. multi-tasking) (Dadda & Bisazza, 2006; Hunt et al., 2006; Rogers et al., 2004). It has widely been studied in birds, reptiles, mammals, fish, and amphibians (Bisazza & Brown, 2011; Rogers et al., 2013). In humans, for example, lateralised behaviour manifests as handedness, and in fish, for visual tasks associated with foraging, predator-escape, predation and conspecific recognition (Bisazza & Brown, 2011; Clotfelter & Kuperberg, 2007; Takeuchi et al., 2010; Takeuchi et al., 2016). An example of behavioural lateralisation in fish has been shown with preferential eye use in zebrafish (*Brachydanio rerio*) (Miklosi et al., 1997).

Hemisphere specialisation has advantages and disadvantages. It is thought that cognitive ability (e.g. reaction time) would be reduced through both competition between hemispheres in the absence of asymmetrical brains, and the duplication of tasks in both hemispheres (Rogers, 2000; Vallortigara et al., 1999). Division of two concurrent visual behavioural tasks between brain hemispheres (e.g. foraging and predator avoidance), therefore logically provide fitness benefits via multitasking (Levy, 1977), reducing neural processing time, and ultimately hastening decision making. Evidence of enhanced ability via simultaneous task processing was shown in domestic chick (*Gallus gallus domesticus*) (Rogers et al., 2004).

Lateralisation, during visual tasks, influences schooling behaviour (Bisazza & Dadda, 2005). Whilst strength and direction (left or right) of laterality vary greatly amongst individuals within groups, there is directional stability within fish populations (Bisazza et al., 2000; Vallortigara

& Rogers, 2005), although sex differences in direction of asymmetry exist in species of poeciliid (Bisazza et al., 1998). Therefore, a single group of fish will adopt directional bias at population level, even though individuals within the group possess different preferences. Laterally placed eyes on the head of a fish divides its visual world into two halves, each projecting almost entirely into the contralateral hemisphere, with very little visual overlap to enable simultaneous use of both eyes for observing stimuli (Bisazza & Brown, 2011; Vanegas & Ito, 1983). Essentially, this means an object can only be processed by one eye/hemisphere at any given time. For example, a fish with a right eye bias for optimal prey detection and a left eye focused upon conspecifics may position itself on the right hand side of the school and therefore, enhance foraging success. This is where lateralisation becomes important in schooling behaviour as it allows fish to position themselves for specific visual tasks (e.g. foraging, predator recognition/avoidance) according to individual bias.

Development of lateralised behaviours is strongly correlated to both genetic and epigenetic (environmental) factors (Vallortigara & Rogers, 2005; Vallortigara et al., 2011). This is shown in zebrafish (*B. rerio*) with the triggering of behavioural asymmetries in response to light exposure (Budaev & Andrew, 2009), and nodal signalling pathways during early development (Halpern et al., 2003). Interestingly, evidence also suggests a genetic link to laterality with *situs inversus* (reversal) of brain asymmetry and associated behavioural lateralities in zebrafish larvae (Barth et al., 2005). Understanding the impact of both genetic and environmental factors on development of asymmetrical behaviours in fish would greatly improve general knowledge of the variation seen in function traits, for example schooling behaviour.

The optic lobe (OL, also known as the tectum) is a neural structure, which, among other things, plays an important role in sensory processing of visually mediated behaviours in fish (Springer et al., 1977). Reddon et al. (2009) found that in cichlid fish brains, strength of habenulae asymmetry is correlated with strength of lateralisation in detour behaviour. To date there is no

known quantitative research in teleosts specifically investigating the relationship between ontogeny of OL asymmetry in teleosts, and strength and direction of behavioural laterality.

Yellow-eyed mullet (*Aldrichetta forsteri*) are an abundant estuarine schooling species with a geographical distribution extending over a large latitudinal and temperate range incorporating coastal waters (to depths of 50 m (Morrison et al., 2014)) off Australia and New Zealand coastlines (Curtis & Shima, 2005; Paulin & Paul, 2006). Life expectancy is around seven years, they school in large aggregations, are omnivorous (diet includes benthic detritus, small invertebrates, and algae) (Morrison et al., 2014), and from a fisheries perspective are considered a minor New Zealand commercial fishery at <50 tonnes annually (Taylor & Paul, 1998). Their abundance makes them a dominant species in New Zealand coastal ecosystems (Curtis & Shima, 2005; Morrison et al., 2014). Factors driving plasticity and development of teleost behavioural traits (e.g. schooling), especially at a phenotypic level, are of growing importance to New Zealand ecosystems and fisheries management (Conrad et al., 2011; Morrison et al., 2014). This body of work aims to improve existing knowledge on the synergies between visual lateralisation and schooling behaviour by investigating the relationship between behavioural lateralisation, optic tectum asymmetry, and schooling behaviour in yellow-eyed mullet.

6.3. Materials and methods

6.3.1 Animals

Experiments were undertaken at The New Zealand Institute for Plant & Food Research's Seafood Research facility in Nelson, New Zealand. Experimental fish were randomly sampled from populations of juvenile (age ~1–2 years) and adult (age ~2–3 years) yellow-eyed mullet (n=50 of each age class), wild caught from the Nelson Haven (41.254°S, 173.278°E) in December 2014. Yellow-eyed mullet are sexually mature once reaching lengths of > 220 mm

(Webb, 1973a). Fish were held in aerated 5000 L flow through tanks, using filtered seawater pumped ~30 L min^{-1} from the surrounding Nelson Haven and water chemistry maintained at ~pH 7.6, 35–36 PSU and DO >90%. Mean nominal ambient seawater temperatures over a 12 month period ranged between 9-21° C and during the experimental period June to August 2016 temperatures ranged between 9–12° C. Both groups were acclimated to tank rearing conditions before testing, for a minimum period of 17 months, at stocking densities not exceeding 15–25 kg^{-1} m^3 and fed a Skretting® diet (Nova ME, Skretting, Australia) twice daily calculated at 2% body mass per day. Size characteristics at the time of study were: mean ± SD mass and fork lengths (FL) 67.9 ± 20.7 g, 174 ± 16.4 mm (juvenile yellow-eyed mullet), 233.3 ± 33.8 g, 252 ±16.8 mm (adult yellow-eyed mullet). These two cohorts of fish were deemed to be of two different age classes based upon length at age criteria defined for the same geographic stock, as described in Curtis and Shima (2005).

6.3.2 Visual bias

Tests of visual bias of a food stimulus were conducted with a choice tank apparatus (dimensions 1500 mm L x 500 mm D x 400 mm W, Fig. 1) following a modified version of the t-maze design used by Roche et al. (2013). Two weeks immediately prior to the experiment, fish were acclimated to viewing food in their home aquarium, administered twice daily via a similar tube to that used in the experiment as described below. Fish were lightly anaesthetised (20 ppm AQUI-S®) before transfer from their home aquarium into the choice tank. Fish were acclimated for 12 h overnight prior to experiments in order to reduce possible stress effects associated with introduction to a novel environment immediately prior to experiments. They were then held separately in a holding pen attached to the choice tank and each individual was gently introduced to the choice tank at the beginning of the experiment through an adjoining door between the holding pen and choice tank to minimise stress. Fish were then placed centrally at the beginning of the runway using a dip net (Roche et al., 2013) and gently encouraged to swim

towards the opposite end where they were presented with, and visually investigated (i.e. did not feed), a food stimulus and were recorded as using either the left or right eye before exiting the runway. Food was administered via a transparent, colourless, water-filled feeding tube (~5 cm diameter) located just beyond the runway exits, isolated from the main water-body and located behind a clear Perspex® barrier positioned at a perpendicular aspect, so as to remove any possible input from the fish's olfactory senses and to force the fish to exit the runway to the left or right. Three series of 10 concurrent measurements of preferential eye use were taken with a five min interval period between each series. These were used to determine direction and strength of lateralisation using a laterality index calculated with the following formula used by Bisazza et al. (1997):

$$\text{Laterality index} = \left[\frac{R-L}{R+L}\right] * 10$$

A positive score indicated right eye preference for viewing food stimuli, negative left eye and zero a nil bias. The closer towards 10 in either direction the stronger the bias. The threshold for strongly biased fish was set at $\geq 6, \leq -6$ representing a minimum 80% bias in either the right or left eye respectively. Fish were considered weakly biased between 0 and 6 and/or 0 and -6. Fish meeting the strongly biased threshold were lightly anaesthetised (20 ppm AQUIS®) and tagged using coloured 'T-bar tags' (Floy®, Floy Tag Inc., WA, USA) inserted anterior to the dorsal fin, for identification in the schooling experiment (para 6.3.3).

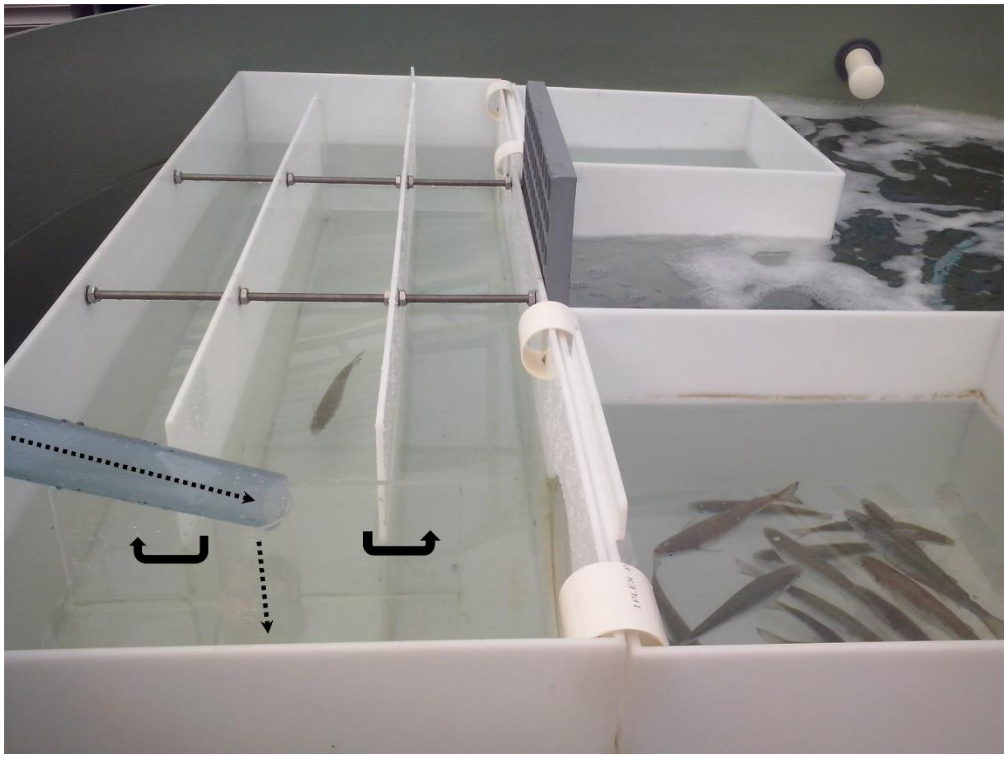

Figure 1 An image of the choice tank apparatus showing a fish swimming down the centre runway towards a feeding tube (marked with dotted arrows) administering a food stimulus for visual inspection. Exits marked with solid arrows. Holding pens visible to the side of the tank.

6.3.3 Spatial positioning during schooling

Following assessments of visual bias using the choice tank apparatus, schooling behaviour was analysed in strongly lateralised left (L), right (R) and control (non- or weakly lateralised (NL)) groups of yellow-eyed mullet to assess whether strongly lateralised fish have a schooling advantage over NL (control) fish, regardless of age. The groups were: (a) n=3 adult R, (b) n=1 juvenile left L and R, and (c) n=1 control (NL juveniles). Each group consisted of 15 individuals, using fish from the choice tank experiment, comprising five tagged for identification as strongly lateralised (L/R), or NL and all remaining 10 fish were untagged NL fish. Given there were only three juveniles out of n=50 juvenile fish tested in the choice tank experiment displaying a strongly lateralised L eye bias, this small number of fish was combined to form one juvenile group of n=3 L, n=5 R eye, and n=7 NL fish (as described in (b) above). Individuals were acclimated overnight in a 13,000 L observation tank (dimensions: diameter

3.5 m, depth 2.6 m, similar seawater quality parameters as para 2.1). The fish immediately formed a school and swam continuously around the tank (termed a rotation). Rotational bias was anti-clockwise for juvenile and clockwise for adult fish. The following morning schooling behaviour was recorded for 30 min (GoPro® Hero3+silver, 1080p resolution, 60 frames per second, medium field of view) from directly above the tank. The percentage of time that each tagged individual occupied an exposed position in the school (quantified as the position closest to the unoccupied area towards the centre of the tank and with no neighbouring fish in that direction) was recorded based on n=30 repeated swimming rotations of the tank (replicates) by each free swimming school (approximately 15 s intervals).

Rotational swimming preference was tested for a period of one month in a 5000 L (2.5 m diameter) tank whereby, 30 juveniles (anti-clockwise preference) were placed together with 50 adults (clockwise preference) to identify whether the initially observed directional biases were plastic, or fixed, responses.

6.3.4 Optic lobe anatomical studies

Yellow-eyed mullet were anaesthetised by lethal overdose (50 ppm AQUI-S®) and mortality confirmed by nil gill movement or reflex response. Whole brains were dissected in preparation for anatomical studies following a modified method by Jozet-Alves et al. (2012) (Fig. 2a,b). Groups consisted of strongly left eye bias (n=3 juvenile), right eye bias (n=9 adult), and control (nil bias n=5 mixed juvenile and adult). Following dissection, brains were immediately fixed for 16–24 h in 4% formaldehyde, 0.1 M phosphate buffered solution (PBS), at pH 7.4. Tissue was then rinsed three times in PBS, placed in a 30% sucrose and PBS solution overnight to protect the tissue, then frozen in liquid nitrogen and stored at -80° C until required for microtome cryosectioning (Starlet 2212, Bright Instrument Company Ltd., England). Optic lobes (OLs) were sectioned at 20 μm thickness and tissue from every 5th section was

photographed (Panasonic DMC-FZ70 digital camera 60 x optical zoom) *in situ* in the frozen embedding medium at around -20 °C (Tissue-Tek®) (Fig. 2c).

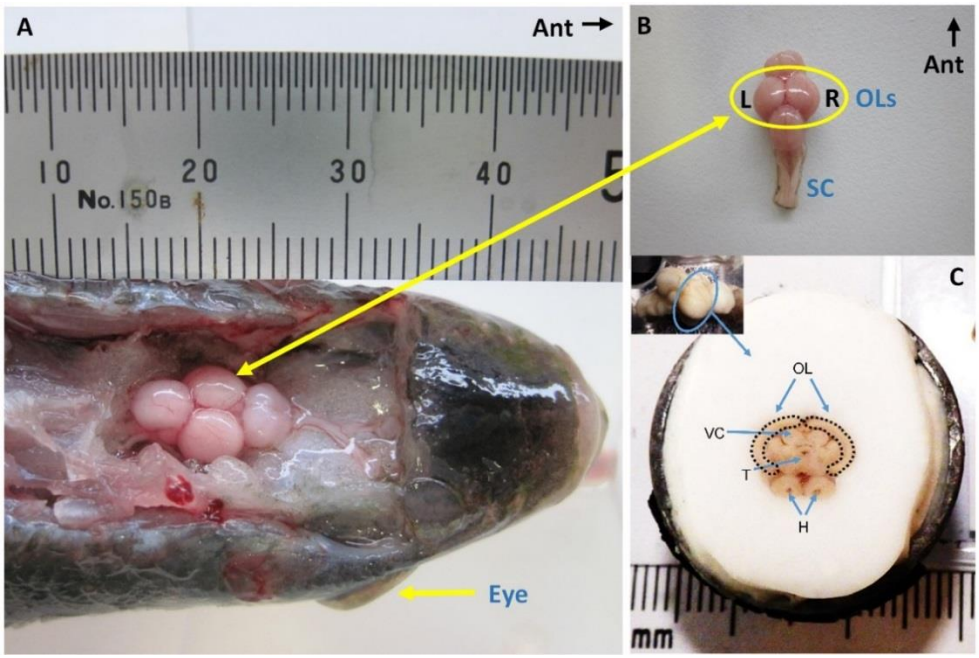

Figure 2 Yellow-eyed mullet brain (a) *in situ* and (b) dissected showing position of left and right optic lobes. Frozen brain sample (c, inset) and transverse cross-section with the optic lobes marked by the dotted line. L, left; R, right; OL, optic lobe; SC, spinal cord; ant, anterior; VC, valvula cerebelli; T, tegmentum; H, hypothalamus.

Images were analysed using ImagePro® Premier 9.0 in order to calculate and compare the area (mm^2) of left and right OLs in individual fish with a left or right eye bias regardless of age (i.e. not adult vs juvenile). Brain structures were identified according to Meek (1983). Clearly defined edges separated the OLs from other brain structures making it possible to accurately measure the areas of interest. Relative volume of the left and right OLs was estimated using the Cavalieri method (Gundersen et al., 1988):

$$V(structure) = t \times \sum a$$

Where t is the known distance between parallel sections and a is the area of each section.

The coefficient of variation (error) of volume estimates was 3.3% (determined from 10 repeated measurements of area using the same OL).

6.3.5 Statistical analysis

Analysis was carried out using R v.3.3.2 or SigmaPlot v.12.5. Laterality data were analysed using a generalised linear binomial mixed model with group (juvenile/adult) and fish number as fixed and random effects respectively (using the lme4 package in R). Schooling position data was analysed using a generalised linear loglinear mixed model with group as a categorical predictor as a fixed effect and replicate as random effect. Comparison of OL volume was analysed by calculating a ratio (volume OL_{left}/OL_{right}). One-sample t-tests of the mean OL ratios, and laterality index (one-sample signed rank test for juveniles after failing assumptions), were used to estimate departures from one. Significance was accepted at $P \leq 0.05$.

6.4. Results

6.4.1. Visual bias of food stimulus

Strongly lateralised juvenile fish were represented by 18% of the population (n=50) with a split of 12% right and 6% left eye bias (Fig. 3). In adults, there were no strongly left eye biased animals while strongly right eye biased fish were represented by 30% of the total population. There was a statistically significant difference between the mean laterality index of the sampled (3.3) and hypothesized (1.0) population in adult fish ($P < 0.001$), but not juveniles ($P = 0.791$). Adult yellow-eyed mullet displayed a weak right eye bias at population level (laterality index of ~3). However, individually they were mostly right eye biased (laterality index > 0), with only eight (16%) out of 50 fish having a weakly left eye bias (laterality index between 0/-5). Four fish (8%) returned a 0 (nil bias) laterality index score. In terms of the population level means of our samples, adult mullet had a significant right eye bias in comparison to juveniles, which had close to nil bias ($P < 0.001$) (Fig. 3). Strength of bias in juveniles ranged between -10 and 9 with ~60% showing a degree of left eye bias, whereas adults ranged between -4 and 10 and a ~76% right eye bias.

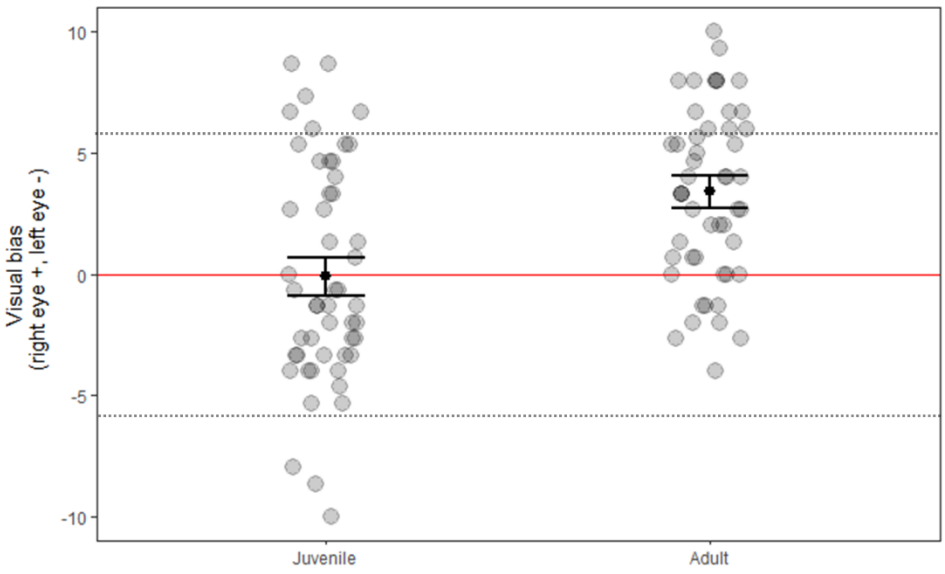

Figure 3 Individual visual laterality for juvenile and adult yellow-eyed mullet (n=50 each). Positive scores indicate a right eye bias, negative a left eye bias. Dotted lines represent strong bias (> 6 and < -6). Data points represent the raw data. The error bars are the 95% CIs around the predicted means.

6.4.2. Schooling position and directional swimming behaviour

Strongly lateralised adult R (17%) and juvenile L & R fish (10% and 22% respectively), on average, spent lesser amounts of time in the exposed area of the school compared with the control group (25%). The greatest difference was found in juvenile left eye bias fish (~2.5-fold) (Fig. 4); however, likely due to the small sample size, the size of the effect was not statistically significant. It is worth noting that during the routine holding of juvenile and adult yellow-eyed mullet, the two age classes showed fixed and opposing directional swimming preferences in separate and identically set up tanks (personal observation). Attempts to alter the directional preferences of juveniles were made by combining the two populations to investigate whether juveniles would assume the directional characteristics of adults over a one month period. During this time, juveniles showed mixed directionality (i.e. were mostly seen schooling with the adult fish, but occasionally formed a separate school of opposing directionality in the centre of the tank). However, when returned to their own tank juveniles resumed their original directional anti-clockwise swimming tendencies (results not presented).

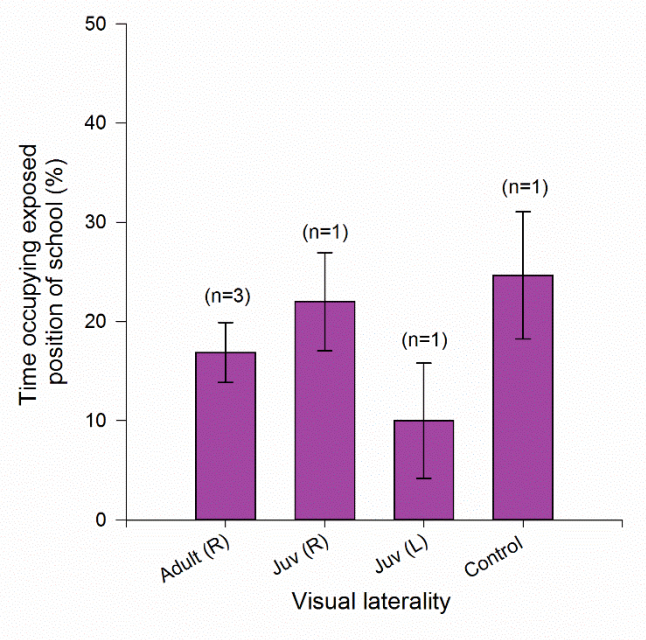

Figure 4 Percentage time spent in the exposed position within schools of strongly lateralised juvenile and adult yellow-eyed mullet. Control group consists of non-lateralised fish. Data represent means with 95% CI. Numbers in brackets indicate number of replicate groups. Significance was accepted at $P \leq 0.05$.

6.4.3. Optic lobe anatomical studies

A ratio of estimated volume of the left and right OLs in L or R eye bias yellow-eyed mullet was calculated from departures from 1. This revealed a size difference between the two lobes for fish with a right eye bias, and a very small difference for left eye and control fish (Fig. 5). Control fish showed a significant difference between the mean of the sampled population and the hypothesised population mean for right eye bias only (right eye bias; T = -2.328, DF = 8, P = 0.048; control P = 0.547, no p-value for left eye bias). Of a population of 50 sampled fish for each size class, only three fish presented with a strongly left eye bias (juvenile) and therefore, the sample size was too small to statistically analyse.

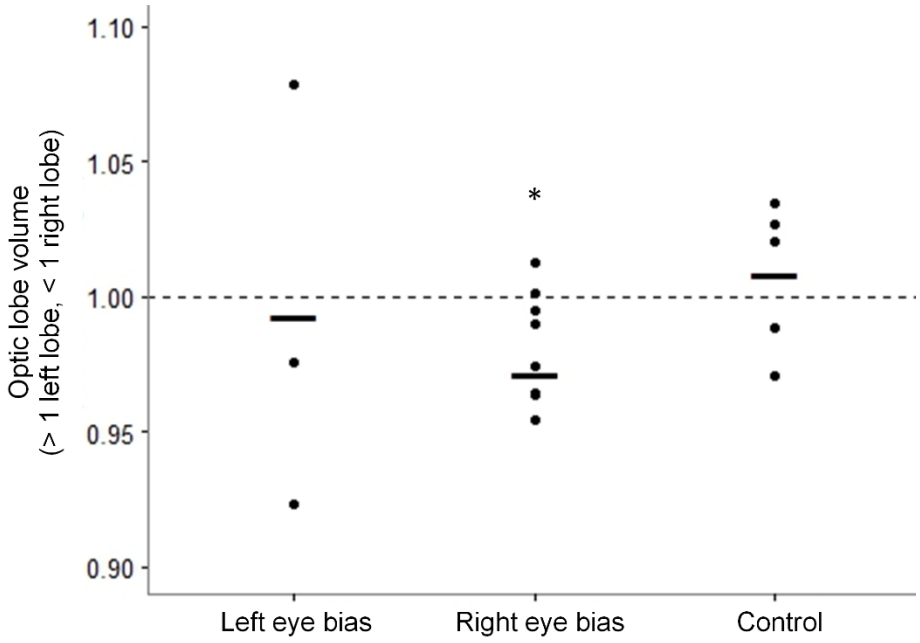

Figure 5 Yellow-eyed mullet optic lobe asymmetry with treatment group ratios > 1 representing larger left lobe and < 1 larger right lobe. Asterisk represents significant differences accepted at $P \leq 0.05$. Data points represent individual fish and the group means are represented by solid lines.

6.5. Discussion

We studied lateralisation of visual functional behaviours in yellow-eyed mullet to identify the various characteristics which support schooling behaviour. The aim of the current study was to also examine direction of visual laterality between juvenile and adults and to discuss the likely role that laterality plays in both individual and group behaviour. Results indicate a level of plasticity in visual laterality between juvenile and adult fish which may suggest selective pressure from environmental and/or adaptive learning on ethology.

6.5.1. Visual bias

Adult yellow-eyed mullet had a mean population laterality index of 3.3, with substantial skew of laterality scores towards positive values (e.g. right eye bias), ultimately equating to ~76% right eye bias for the group as a whole. Interestingly, juveniles showed an almost equal number

of left and right biased individuals (laterality index of -0.2, 0 being nil bias), and no significant difference in laterality between individuals (1-sample t-test). This suggests that response to visual stimuli is either inconsistent in this age group at a population level, or individuals were undergoing an ontogenetic shift, which would suggest direction of lateralisation is age dependent. The fact there is a difference between visual bias in juvenile and adult fish seems to suggest an ontogenetic shift; one from no preference in naive juveniles, to a clear preference in older and larger individuals. Results showed that only 8 out of 50 adult fish (16%) had a left turning bias in the choice test, which may indicate that the left hemisphere (right eye bias) is specialised for investigating food stimuli.

Previous research by Bisazza et al. (2007) reported population bias in groups of mixed laterality fish to be around 65–70%, consistent with results from adult yellow-eyed mullet in the current study. Further research is required in order to identify whether current results have developmental origins. An alternative hypothesis is that no ontogenetic effect is present and the observation of more lateralised individuals in older wild caught/captive yellow-eyed mullet is a result of increased fitness and survival of lateralised juveniles. Therefore, lateralisation appears more common in older age classes (i.e. non-lateralised individuals had a lower chance of survival and therefore year-class progression at younger life-stages). Previous studies have shown that many species display a high degree of variation in both strength and direction (left or right bias) of visual lateralisation amongst individuals, but at a population level within fish schools there is directional stability (>50% population bias) (Bisazza et al., 2000; Vallortigara & Rogers, 2005). For example, Bisazza et al. (2000) found a 62% turning bias from 16 species of fish across 13 families.

6.5.2. School position and directional swimming behaviour

In all schools, strongly lateralised (> 80% bias) fish tended to spend less time in the exposed position of the school than the NL yellow-eyed mullet and this was considerably less in juvenile (L) fish, although the difference was not statistically significant in any of the three groups; a result considered likely due to the small sample size. When considered in terms of the advantages schooling behaviour confers to individuals (e.g. decreased predation risk (Bisazza & Brown, 2011)), these results suggest that fish with developed preferential eye use, and that occupy positions of safety more frequently, have a lower risk of predation than fish with arbitrary spatial positioning. This is consistent with previous research showing that fish position themselves in specific areas in order to maximise fitness potential, including reduced energy costs associated with swimming (Marras et al., 2015), and decreased predation risk (Larsson, 2012). It is important to note that a 'tagging effect' may have been introduced within the experimental design, given only the focal fish were tagged and therefore, observed behavioural responses may not solely be due to lateral preferences. However, we believe this tagging effect to be minimal as the control group spent only subtly less time in the exposed area of the school than if it had been a random occurrence. Schools of yellow-eyed mullet (particularly juveniles (Higham et al., 2005)) inhabit the frequently rich feeding grounds of estuaries (Morrison et al., 2014), exposing them to predators from both sea and air (avian) (de Carvalho et al., 2007) and are also exposed to tidal rhythms. Given this continuous and often considerable environmental change, behavioural laterality could play an important role in optimising schooling behaviour in juvenile and adult fish.

6.5.3. Optic lobe anatomical studies

It was hypothesised that the direction of visual bias (L or R) in strongly lateralised fish (≥ 80% bias) is correlated to OL size differences in the opposite brain hemisphere, and we did indeed find size differences between L and R OLs. However, this was only evident in fish with a R

eye bias having a larger R OL, but was not matched in L eye biased fish, and intriguingly these results were contrary to what we expected to find in the direction of hemisphere differences (i.e. R eye bias = larger L OL). The assumption made during the choice test experiment (individual laterality) was that the fish were conducting a single visual task and using their dominant eye to investigate a food stimulus. It is possible, but not tested in the current study, that they were in fact processing two concurrent visual tasks; escape and investigating a food stimulus. This being the case, and if the escape task was a higher priority, then it is possible that what was recorded as the dominant eye viewing a food stimulus, was actually conducting a secondary task. Hierarchical decision-making is common amongst species (Gueron et al., 1996) and this would be an interesting area for further research to test this theory.

Interestingly, and secondary to our study aim in identifying size differences in OL morphology in strongly L and R lateralised fish, it appears that differences may exist between adult and juvenile yellow-eyed mullet. However, as our study design did not allow us to test this theory, it is currently unknown whether differences between the juvenile and adult samples tested is a developmental phenomenon (ontogenetic), or otherwise. In combination with our observations of visual biases, results show that within the sampled population, juvenile and adult individuals have different behavioural tendencies, but it is unknown if this correlates with differences in OL asymmetry and this would be an interesting area for future research. What little is known about the correlation between visual lateralisation and the size of brain structures (including OLs) has been studied in *Sepia* (Jozet-Alves et al., 2012), and habenular nuclei in cichlid fish (Gutierrez-Ibanez et al., 2011). In both cases strong correlations were found. To the authors' knowledge there are no published anatomical studies of OL volume or the functional implications of brain structure on behaviour (e.g. schooling) in any teleost species.

6.5.4. Ontogenetic plasticity and visual lateralisation

Differences in population level laterality shown between juvenile and adult yellow-eyed mullet (nil vs R eye bias respectively) may relate to their life history strategy. Schools of juvenile yellow-eyed mullet start life in nurseries of shallow estuarine waters, moving to deeper coastal waters (up to 50 m) as adults (Higham et al., 2005; Morrison et al., 2014). Conceivably visual specialisation could therefore, be closely related to epigenetic (environmental) factors resulting from habitat change. Other relevant factors could include changes in prey selectivity with age (and habitat change), interspecies interactions (e.g. potentially including larger and different predators). Plasticity, related to growth, may support fish to adapt visually mediated behaviour to suit emergent ecological niches.

Along with spatial positioning within a school, lateralisation of rotational swimming bias is also a common behaviour in fish species. For example; mosquito fish (*Gambusia holbrooki*) (Bisazza & Vallortigara, 1996), and sturgeon (*Acipenser ruthenus*) (Izvekov et al., 2014). Similarly, some indications of rotational swimming preferences were evident in yellow-eyed mullet, as observed by the rotational swimming preferences between juvenile and adult groups. Given that juveniles showed no adaptive behaviour as a result of a forced opposing directional swimming, this suggests a fixed directional preference in younger fish. Further work confirming this observation would likely strengthen the association between lateralisation and rotational swimming in this species. One of the factors believed to influence rotational bias is cerebral asymmetry. Three likely drivers of this and other lateralised schooling behaviours include ontogenetic plasticity, hereditary and environmental factors.

Animal behaviour is known to be intrinsically linked with both heritability and adaptive responses to environmental selection pressures (Bell, 2005; Corballis, 2009). There is a limited but growing body of work on the genetic basis for lateralisation of behaviour in schooling fish,

including shoaling, boldness and aggression behaviours in sea bass (*Dicentrarchus labrax*) and zebrafish (*Danio rerio*) (Faucher et al., 2010; Wright et al., 2003). Improved, and relatively inexpensive, sequencing technologies (Boake et al., 2002), provide the opportunity for future research to investigate the interactive effect of genes and the environment on behavioural traits in many species and this is an important area for future research.

Given that yellow-eyed mullet ecology includes migration of fish from shallow estuarine ecosystems as juveniles, to deeper coastal waters as adults, (Morrison et al., 2014), we suggest that environment is likely to be closely related to gene expression of behavioural traits (e.g. development of laterality). In support of this, it has been shown in individual zebrafish that *situs inversus* (reversal of anatomical asymmetry) results in the reversal of some, but not all associated behaviours (Barth et al., 2005), which would suggest environmental factors can play a strong role in the direction of asymmetry in other species. It is also possible that the differences between juvenile and adult laterality in our study may also be linked to factors related to ontogeny. Much is yet to be learned about the drivers of plasticity in behavioural traits such as laterality.

6.6. Summary

The authors found that adult fish show a consistent right-eye bias, but not juvenile, and that strongly R eye biased fish have larger right optic tecta. Lateralisation was varied in the direction (left or right) and strength of visual bias in individual yellow-eyed mullet, however at a population level there is a significant bias in adults. Results also indicate that schooling behaviour may be related to these behavioural biases towards a food stimulus with strongly lateralised fish tending to occupy positions of safety within a school more often than non-lateralised fish. The age difference in lateralisation suggests the need for further research in this area and in particular, it would be interesting to monitor ontogenetic changes in laterality and schooling behaviour within individuals. It is likely there is a level of adaptive and/or

phenotypic plasticity in rotational swimming bias, and of laterality in general, that is possibly related to both genetic and environmental factors. Understanding the factors which underpin species ontogeny and plasticity of phenotypic behavioural characteristics could provide direct benefits for improved fisheries management of these valuable coastal ecosystems.

6.7. Ethics statement

All experiments were conducted in accordance with the University of Canterbury Animal Ethics Committee (ref: 2016/06R). Methods were carried out in accordance with approved guidelines.

7 Morphology and hydro-sensory role of superficial neuromasts in schooling behaviour of yellow-eyed mullet (*Aldrichetta forsteri*)

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Middlemiss, K.L., Cook, D.G., Jerrett, A.R., Davison, W., (2017). Morphology and hydro-sensory role of superficial neuromasts in schooling behaviour of yellow-eyed mullet (*Aldrichetta forsteri*). Journal of Comparative Physiology A, 1-11.

7.1. Abstract

The lateral line system is a mechanosensory organ found in all fish species and located on the skin or in subdermal canals. The basic functional units are superficial and canal neuromasts, which are involved in hydrodynamic sensing and cohesion in schooling fish. Yellow-eyed mullet (*Aldrichetta forsteri*) are an obligate schooling species found commonly in shallow coastal areas of New Zealand and Australia. Schooling is a fundamental part of their behavioural repertoire, yet little is known about the structure or functionality of the lateral line in this species. We used scanning electron microscopy to characterise the morphology of trunk superficial neuromasts. We then took a multi-sensory approach and conducted behavioural experiments comparing school structure in groups of fish with and without fully functioning lateral lines, under photopic and scotopic conditions. A highly developed hydro-sensing system exists on the trunk of yellow-eyed mullet consisting of superficial neuromasts containing hundreds of hair cells aligned, with respect to their most sensitive axis, in a rostrocaudal direction. Without functioning superficial neuromasts, schooling behaviour was disrupted under both photopic and scotopic conditions and their ability to detect stationary objects decreased. Results highlight the importance of this component of the lateral line system to schooling behaviour.

7.2. Introduction

Fish use multiple senses to evaluate the aquatic environment including visual, chemosensory (olfaction and gustation) and octavolateralis (lateral line, hearing and vestibular) systems (Montgomery & Carton, 2008). The lateral line system (LLS) is unique to the aquatic environment and is found in all fish species and many amphibians (Coombs et al., 1988; Montgomery et al., 2014; Webb, 2011). It consists of mechanoreceptors in the skin called canal neuromasts (CNs) and superficial neuromasts (SNs) found on the head, trunk and tail fin of

fish (Blaxter, 1987; Dijkgraaf, 1962). The sensory epithelium of a neuromast consists of many hair cells, each of which carries a ciliary bundle that consists of a single long kinocilium and many stereovilli that are graded in length. The ciliary bundles of the hair cells project into a gelatinous cupula (Webb, 2011). The hair cells of the fish lateral line are innervated by afferent and possibly also by efferent nerve fibres (Bleckmann & Zelick, 2009; Flock & Wersall, 1962; Webb, 2011). Hair cells are directionally sensitive and movement of the cupula, via a hydrodynamic stimulus, generates excitation of the cells and an electrical message is then sent to the brain informing the fish of changes in the surrounding water (Blaxter, 1987).

The lateral line system has been studied extensively in many teleost species including cod, salmon, and cichlids, and is well described by Kasumyan (2003). There is much diversity in morphology due most likely to the variety of habitats occupied by teleosts (Klein et al., 2013; Wellenreuther et al., 2010). In the early 1990s, the lateral line canal system was described in 31 species from 13 genera of the family Mugilidae, to which yellow-eyed mullet (*Aldrichetta forsteri*) belong. In yellow-eyed mullet, superficial neuromasts were located on almost every trunk scale (Liu & Shen, 1993), but were not described. Similar results were found in closely related grey mullet (*Mugil cephalus*) by Ishida et al. (2015), who found a mixture of both types of neuromast on the cephalic (head) region, although data on the latter was not published (personal communication). Song (1981) found a well-developed cephalic lateral line system in 13 species of Chinese mugilid. Mugilidae have developed a highly sensitive mechanosensory system in order to detect hydrodynamic changes, however, nothing is known of the morphology or importance of either of these neuromasts in New Zealand yellow-eyed mullet which school in large numbers in dynamic estuarine environments.

Schooling behaviour is thought to have evolved after the development of the LLS (Larsson, 2009), the functionality of which is known to play a significant role in group dynamics in large aggregates of fish (Gray & Denton, 1991). Ultimately, fish form schools to enhance their

survival (Partridge, 1981b) and around 50% of fish species display this behaviour (Larsson, 2012; Shaw, 1978). Present studies highlighting the significance of the LLS in maintenance of school structure include golden shiner (*Notemigonus crysoleucas*), tuna (*Euthynnus affinis*), tadpoles (*Xenopus laevis*) and firehead tetras (*Hemigrammus bleheri*) (Burgess & Shaw, 1981; Cahn, 1972; Faucher et al., 2010; Lum et al., 1982). The LLS of Mexican blind cave fish has been extensively studied, not only because a complete lack of vision does not deter their ability to detect and maintain inter-individual distances (Partridge & Pitcher, 1980), but also for their ability to discriminate between objects (Von Campenhausen et al., 1981). Our study is the first to characterise the morphology and orientation of trunk SNs in this species and improves our understanding of their role in maintaining schooling behaviour and obstacle avoidance.

7.3. Materials and methods

7.3.1. Animals

Experiments were undertaken at the New Zealand Institute for Plant & Food Research Limited (PFR) Seafood Research facility in Nelson, New Zealand. Fish used in the behavioural experiments were randomly sampled from a population of 200 wild caught yellow-eyed mullet from the Nelson Haven (41.254°S, 173.278°E) in December 2014. Fish were reared in aerated 5000 L flow through tanks, using filtered seawater pumped ~30 L min^{-1} from the surrounding Nelson Haven. Water chemistry was maintained at ~pH 7.6, 35–36 practical salinity units (PSU) and dissolved oxygen (DO) >90%. Mean nominal ambient seawater temperatures over a 12 month period ranged between 9 and 21 °C. During the experimental period March to May 2016 temperatures ranged between 12 and 15 °C. Fish were acclimated to tank rearing conditions for a minimum period of one year after capture and held at stocking densities not exceeding 15–25 kg m^3 and fed a Skretting® diet (Nova ME, Skretting, Australia) twice daily calculated at 2% body mass per day. Nil impact capture techniques, combined with nil handling for at least one month prior to behavioural experiments, were exercised in order to minimise

potential scale/neuromast damage due to handling. The mean fish mass and lengths (± standard deviation) were: 226 ± 31 g, 254 ± 9 mm (control), 219 ± 23 g, 251 ± 9 mm (treatment).

7.3.2. Neuromast morphology

Characterisation of SN morphology and confirmation of neuromast ablation post behavioural experiment (para 7.3.4) was conducted using scanning electron microscopy (SEM). On completion of schooling experiments (9 days post-treatment) (para 7.3.4), control (n=5, mean mass, 304 ± 24 g and length 251.2 ± 9.1 mm) and treatment fish (n=3, mean mass 215.4 ± 17 g and length 251 ± 5.1 mm), were randomly selected and euthanised with an overdose of AQUI-S® (AQUI-S New Zealand Ltd.). A 2.5 cm^2 tissue sample was dissected from each fish at the lateral mid-trunk area, posterior to the pectoral fin. Samples were fixed in 4% formalin in a 0.05 M phosphate buffer solution (PBS) (50 mM NaH_2PO_4, 154 mM NaCl, pH ~7.5) and stored at 4° C until required. An initial trial (by the authors) found that sonification of samples was not necessary in order to expose the ciliary bundles for imaging, as the gelatinous cupula covering SNs was destroyed during the fixation process. Samples were post-fixed in 2% osmium tetroxide (OsO_4), washed in 0.05 M PBS for 5 h and then dehydrated using a graded ethanol series (50, 70, 80, 90, 95, 100, 100%) over 2 days at room temperature (22 °C). They were then treated with amyl acetate ($C_7H_{14}O_2$) overnight. A critical point drier (Polaron E3000 series II) was used to remove all moisture from the samples before mounting on aluminium stubs and coating in carbon and gold palladium (~20 nM thickness) prior to imaging.

7.3.3. Neuromast ablation and DASPEI labelling

An initial trial was conducted (using a similar technique described by Van Trump et al. (2010) for complete ablation of both SNs and CNs) to ensure inactivation of the LLS could be achieved with aminoglycoside antibiotic gentamicin sulphate (Fisher Scientific). Treatment fish (n=3, mean mass 8.8 ± 3.3 g, length 98.3 ± 15.9 mm) were placed in seawater containing 0.002% gentamicin for a 24 h period then post-rinsed in seawater. Both treatment and control (no

gentamicin treatment) animals (n=2, mean mass 4.1 ± 0.4 g, length 80.5 ± 3.5 mm), were then placed in a solution of 0.008% DASPEI ((2-(4-(dimethylamino)styryl) -N-Ethylpyridinium iodide, (ThermoFisher) for 1 h under dark conditions in order to stain neuromast cilia. Animals were euthanised with an overdose of tricaine methanesulfonate (MS-222) (Sigma-Aldrich) and SNs immediately examined using fluorescence microscopy to confirm ablation.

7.3.4. Imaging

All fluorescence microscopy and SEM work was carried out at the University of Canterbury to confirm neuromast ablation with gentamicin from the initial trial. Samples were analysed using a fluorescence stereomicroscope (Leica, MZ10F) equipped with GFP Plus filter cube (excitation: 480–490 nM, emission: 510 nM (barrier filter)). Images were captured with a Leica camera (DFC 310 FX) and analysed with Leica Application Suite (LAS) v. 3.6.0 software. Samples for SEM morphological examination (and confirmation of gentamicin ablation of SNs post completion of behavioural experiments) were analysed with a variable pressure SEM (JEOL JSM IT300LV), imaged using JSM IT300 InTouchScope™ software, and morphological measurements made with ImagePro® (Premier 9.0) and Python 2.7.10.

7.3.5. Schooling behaviour

Treatment fish (n=3 groups of 10 fish) were placed in a bath of seawater containing 0.002% gentamicin for a 24 h period to ablate neuromast hair cells, and control fish (n=1 group of 10 fish) received no treatment. Behavioural experiments for the first group began 24 h post gentamicin treatment and observations for all subsequent groups were completed within nine days post-treatment. SEM was later used to confirm ablation of SNs with gentamicin (n=3, para 7.3.3) in fish nine days post-treatment. Each group was given a 24 h acclimation period upon transfer to the experimental tank prior to the experiment. Experimental setup included a 13,000 L flow through circular tank (similarly described in para 7.3.1) equipped with an ARIS sonar (Explorer 3000, Sound Metrics) and calibrated stereo-video camera system consisting of

a pair of Go-Pro® cameras (1080 p resolution, 60 frames per second, medium field of view) at a separation distance of 700 mm and 8° incline. Fish continuously schooled around the tank and were observed at separate times in still water (no flow), under photopic (cameras), and scotopic conditions (sonar). Start times for both camera and sonar recordings were standardised between fish groups and conducted over a 30–40 min period. Sonar recordings were carried out after sunset, however, unavoidably, urban and industrial ambient light was present so reference to scotopic (sonar) conditions should be regarded as 'limited light'.

All sonar and stereo-video imagery was analysed with measurements made using ARIScope (Sound Metrics) and EventMeasure® (SeaGIS, 2017) software in two and three-dimensional space (2D and 3D) respectively. The behaviours of individuals within the schools were investigated using variables: nearest neighbour distance (NND, the distance between each individual to the neighbouring fish in closest proximity), separation angle (SA, a measure of polarity), swimming speed, and obstacle collision rates. Means were calculated from five repeat measurements of separate frames using all 10 fish from each group (i.e. n=50 control, n=150 treatment). Briefly, ARIScope and SeaGIS software were used to measure NND from the mid-point or head of each fish to its nearest neighbour, SA from the angle between the directions of each pair of fish (fish direction defined by the head and tail coordinates), and swimming speed from head positions measured at different time points (i.e. t_1 and t_2). A stationary obstacle, consisting of a colourless and transparent Plexiglass® screen 1.5 m x 300 mm x 3 mm attached to the tank wall, was extended into the tank at a 90° angle for the final ~10 min of the observation period. Collision rates were calculated from the percentage time the school (≥ one individual), was seen to make contact with the obstacle based on the number of passes, as was clearly visible in both sonar and stereo-video footage. Measurement of 1^{st} – 9^{th} nearest neighbours (NND 1–9, i.e. distances between all individuals in the school) in control (n=1) and

treatment (n=3) groups is based on means from n=50 measurements (10 fish per group; 5 repeat measures from separate camera frames) and n=150 respectively.

7.3.6. Statistical analysis

Analysis was carried out using SigmaPlot v.12.5, and Minitab R16 (collision avoidance only) and data are represented as the mean with 95% CI. Significance was accepted at $P \leq 0.05$. Data from schooling behaviour was analysed using a 1-way Analysis of Variance (ANOVA) and a Holm-Sidak test for comparison between control and treatment groups. A Kruskal-Wallis 1-way ANOVA on ranks was carried out on behavioural data failing assumptions (swimming speed) and a Dunn's test used for comparison between treatment groups. A binary logistic regression was used for analysis of the proportional data for the collision avoidance experiment to compare the treatment and control groups within photopic and scotopic conditions. It was not possible to carry out a pairwise comparison between photopic and scotopic datasets due to the two different measurement systems employed (3D (photopic) and 2D (scotopic)). Morphological data was analysed with SigmaPlot v.12.5.

7.4. Results

7.4.1. Neuromast ablation and florescence labelling

Ablation of neuromasts was confirmed using both florescence microscopy and scanning electron microscopy (Figs. 1, 2). Active cells vividly fluoresced on the trunk of control fish (Fig. 1a, b) compared to faintly or non-stained cells in treatment fish (Fig.1 c, d). Structural damage was evident in some of the cilia investigated under SEM with obvious deformity, especially to kinocilia (Fig. 2).

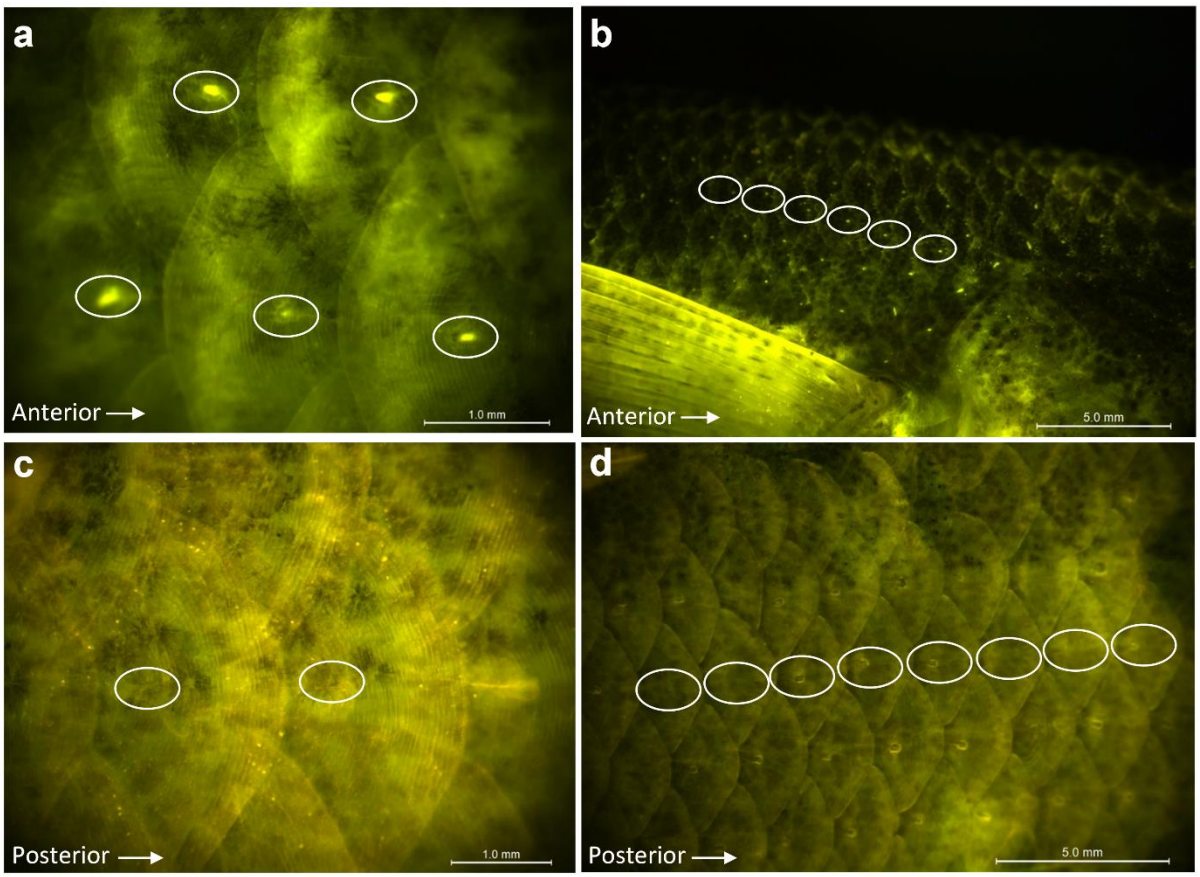

Figure 1 Florescence microscopy images of yellow-eyed mullet (*Aldrichetta forsteri*) trunk lateral line scales each containing a single superficial neuromast stained with DASPEI. Images (a) and (b) are the control group showing the active neuromasts fluorescing (and marked in circles), and (c) and (d) the gentamicin treatment group with inactivated (non-fluorescing) neuromasts (again marked with circles).

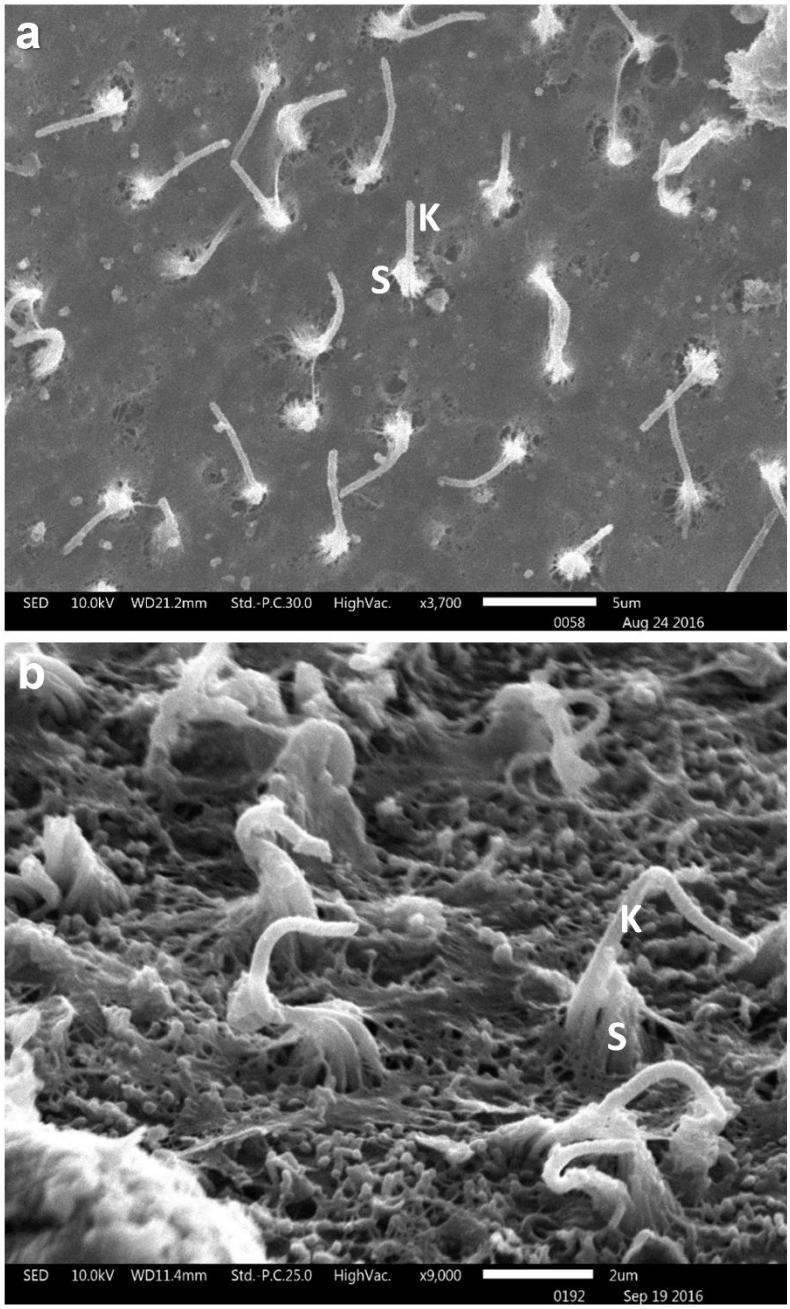

Figure 2 Yellow-eyed mullet (*Aldrichetta forsteri*) trunk superficial neuromasts imaged with scanning electron microscopy showing (a) control (no gentamicin treatment), and (b) damage to ciliary bundles contained within the neuromast after treatment with gentamicin. Abbreviations: K, kinocilium; S, stereocilia.

7.4.2. Neuromast morphology

Morphological terminology used throughout this study is based on the publications of Liu and Shen (1991); Liu and Shen (1993) and (Schmitz et al., 2014). The study region was limited to trunk-based SNs. A single oval shaped SN sits recessed into a shallow groove running laterally

from anterior to posterior along each scale (Figs. 3a, c), and each groove is completely independent from those located on adjacent scales (Fig. 3a). The mean surface area of neuromasts (n=4) is 0.032 ± 0.009 mm^2. Each consists of many hair cells (n=4, mean 275 ± 43 ciliary bundles), and the ciliary bundle of each hair cell consists of a single long kinocilium and several stereovilli of graduated length (Figs. 3d, e), covered by a cupula (not shown). Kinocilia have a mean diameter of 0.29 μm (± 0.02, n=25 (based on five independent kinocilia from five SNs). Ciliary bundles are located centrally on the neuromast combining to form an oval shape (Fig. 3b) occupying a mean area of around 19% (± 2, n=4) of the total surface area. Investigation of cilia spatial arrangement found a mean distance of 3.1 ± 0.05 μm between nearest ciliary bundles (based on n=4 SNs) (Fig. 3d). Direction of polarisation (sensitivity) was determined by identifying the axis between the shortest stereocilium and the kinocilium (Fig. 3e). From the current study it was determined that sensitivity to hydrodynamic stimuli in yellow-eyed mullet runs parallel to the long axis of the SNs (Fig. 3b), which are orientated in a rostrocaudal direction along the body of the fish, and that ciliary bundles within each SN are polarised in opposing directions along the rostrocaudal axis.

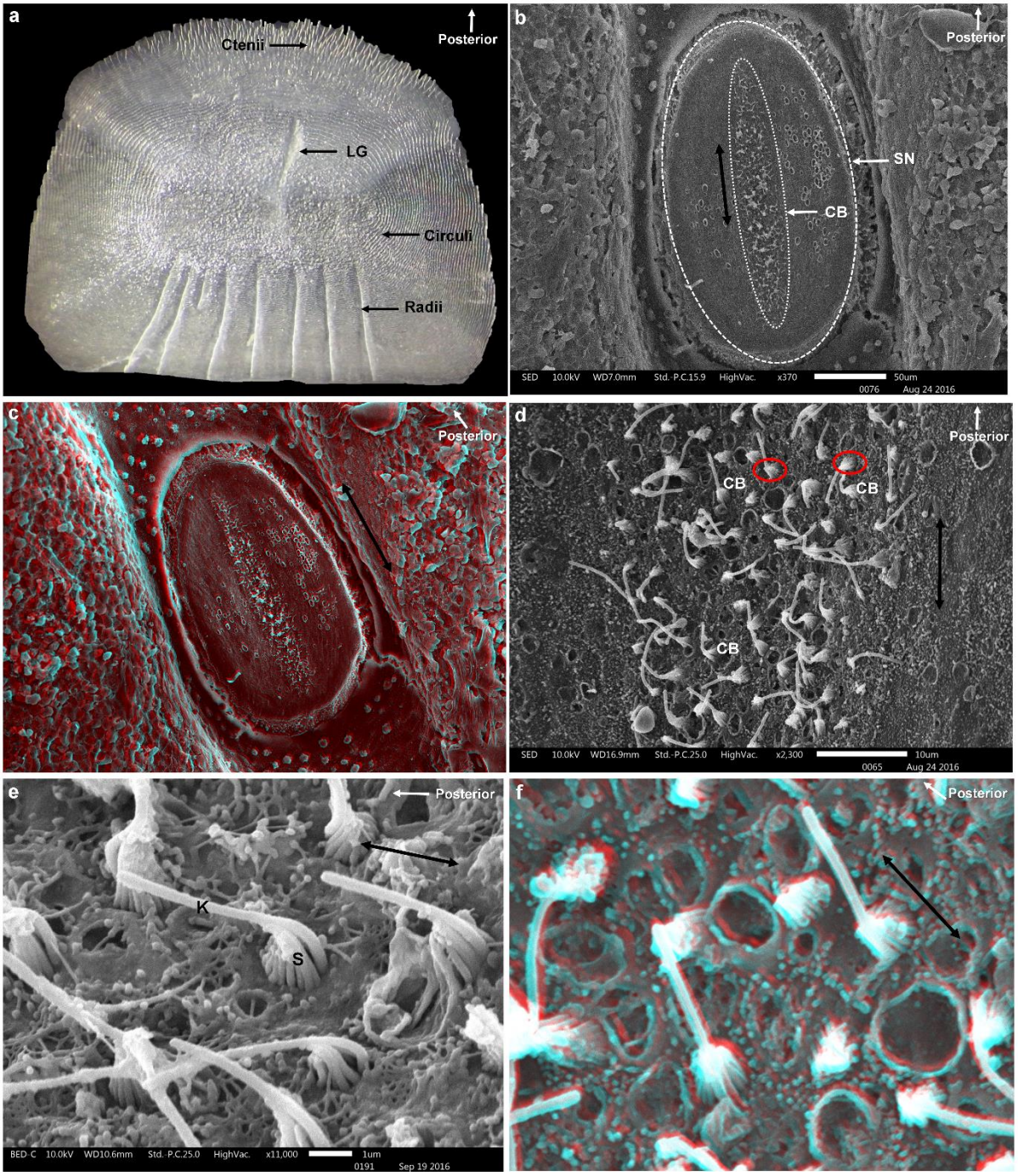

Figure 3 Images of lateral line superficial neuromast cells in yellow-eyed mullet (*Aldrichetta forsteri*). Stereo microscopy: (a); trunk lateral line scale illustrating the location of the longitudinal groove housing a SN. Scanning electron microscopy: (b) superficial neuromast with ciliary bundles (cupula absent) in the longitudinal groove (also shown as a 3D image (c)), (d) multiple ciliary bundles showing kinocilium and stereocilia in different axis of polarisation to each other marked with red circles, and (e) close up of individual ciliary bundles (also shown as a 3D image (f)). Arrows indicate direction of polarity. Abbreviations; LG, longitudinal groove; SN, superficial neuromast; CB, ciliary bundle; K, kinocilium; S, stereocilia.

7.4.3. Schooling behaviour

Yellow-eyed mullet (control and treatment) were observed for changes in school structure under photopic and scotopic conditions. Fish without a fully functioning lateral line system showed disruption to school cohesion when compared to the control group with both the SA and NND significantly reduced in photopic treatment fish ($P \leq 0.001$; $P = 0.028$ respectively), (Figs. 4a, b). SA in this group was reduced from 13.8 ± 2.44° to 11.22 ± 1.07° and the mean NND reduced by about 24% from 248.09 ± 18.32 mm (~ 1.0 body length (BL)) to 189.75 ± 9.21 mm (~0.75 BL), thereby reducing the area of the school. Under scotopic conditions there was very little change in SA between control and treatment groups ($P = 0.122$; 7.51 ± 0.72° (control); 8.40 ± 0.72° (treatment)), however, the NND was significantly increased in treatment fish ($P \leq 0.001$) with mean distances of 310.99 ± 18.16 mm (~1.2 BL) and 377.74 ± 17.07 mm (~1.5 BL) respectively between control and treatment groups (~21% increase). Whilst no pairwise comparison can be made between results from photopic and scotopic data sets, due to the different measuring systems used (i.e. 2D sonar (scotopic) v 3D stereo-video systems (photopic)), it is interesting to note that NND increased under reduced light conditions (scotopic) for treatment group fish whilst, conversely, the distance between fish decreased in treatment fish with full use of their visual sensory system, but no LLS. To test if the structure of the school, in terms of inter-individual distance, remains constant throughout, the mean spacing between fish and their increasing 1^{st} to 9^{th} neighbours was measured in photopic fish. Analysis showed a linear relationship (Fig. 5) in both control and treatment groups fish (multiple $r^2 = 0.76$).

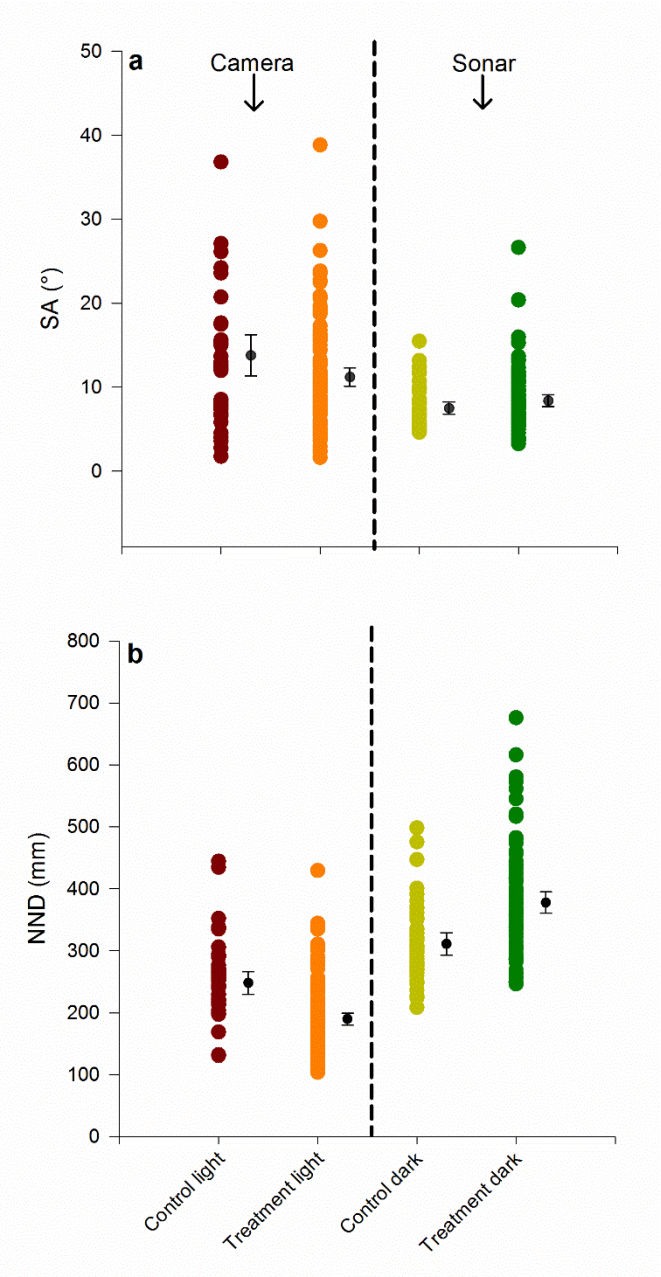

Figure 4 Comparisons between school structure of control and treatment (gentamicin ablation) groups of yellow-eyed mullet (*Aldrichetta forsteri*). Variables of (a) separation angle (SA), and (b) nearest neighbour distance (NND) were measured under photopic and scotopic conditions. Data points represent means and error bars are 95% CI. If the intervals overlap, treatments are statistically different.

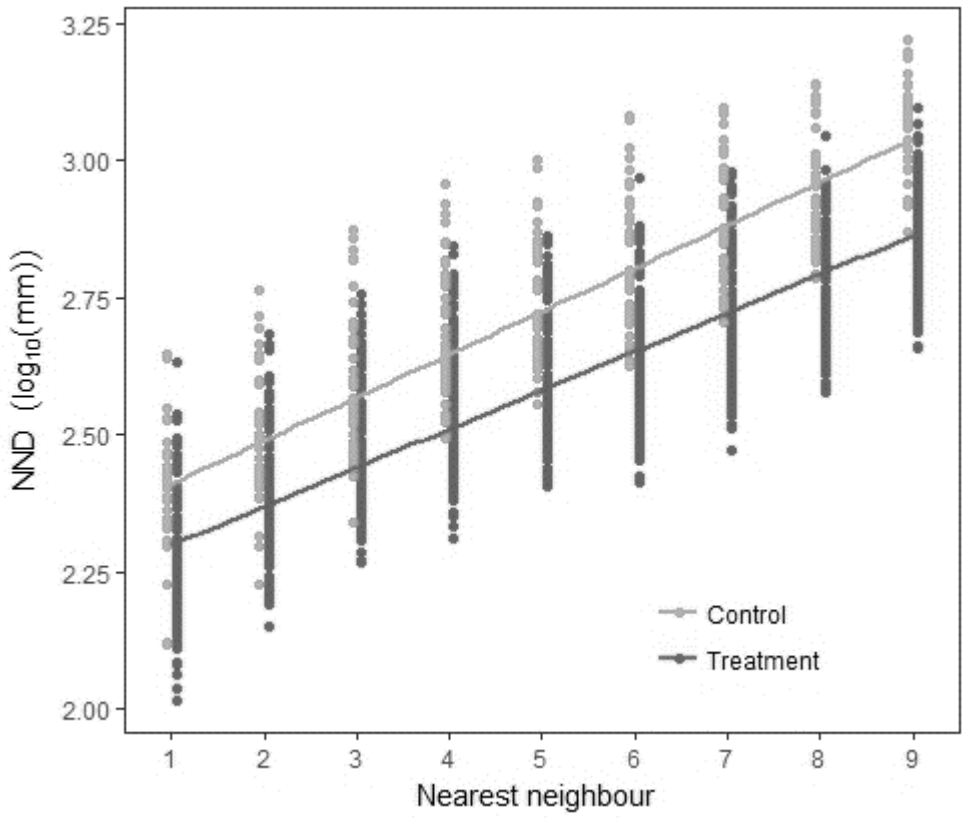

Figure 5 Mean nearest neighbour distances (NND) in both control (n=1) and treatment (inactivated lateral line system) (n=3) groups of yellow-eyed mullet (*Aldrichetta forsteri*) and 1st–9th nearest neighbours.

Swimming speed was also used as a variable to measure possible changes in school structure between groups (Fig. 6). Results showed that under both photopic and scotopic conditions the speed of the school was significantly reduced in the treatment group compared with the control ($P < 0.001$ for both). There was a ~30% reduction in the speed of photopic treatment groups when compared to the control (means of 2.69 ± 0.09 and 3.85 ± 0.09 body lengths s^{-1} (BL s^{-1}; fork length) respectively) while in scotopic treatment groups swimming speed was reduced by ~23% compared with the control group (means of 2.02 ± 0.15 and 2.62 ± 0.13 BL s^{-1} respectively). Whilst no comparison could be made between photopic and scotopic groups for reasons previously explained, it appears that swimming speed was decreased in darkened conditions.

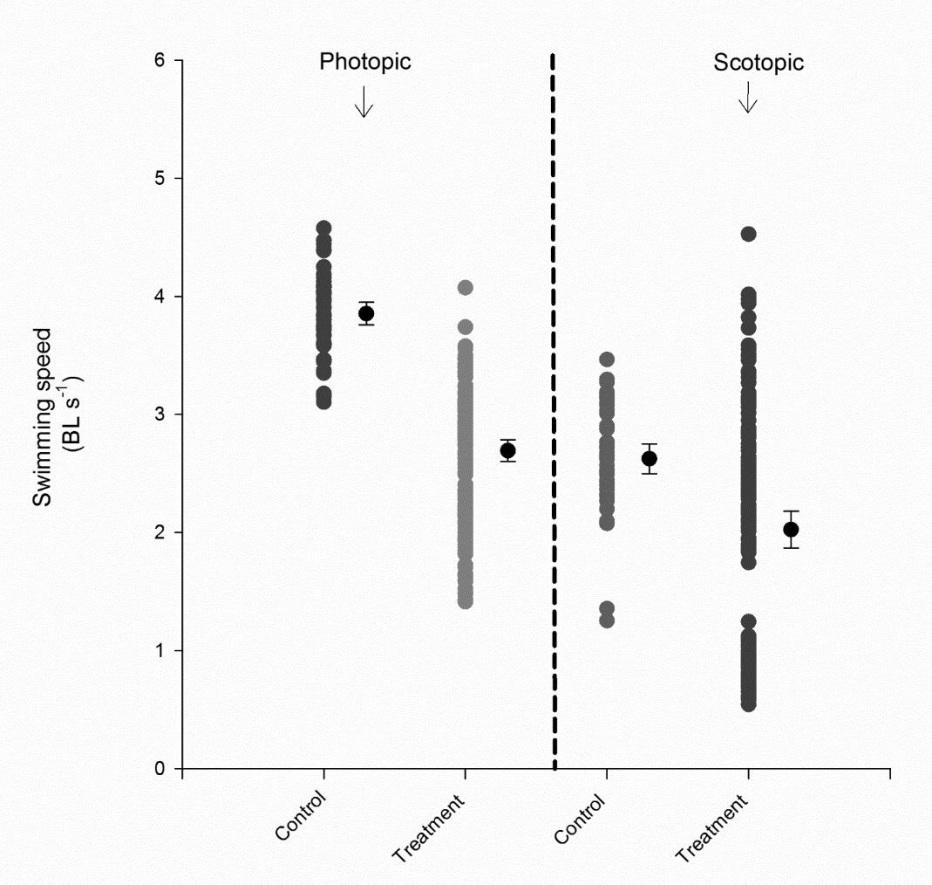

Figure 6 Swimming speed of control and treatment (inactivated lateral line system) groups of yellow-eyed mullet (*Aldrichetta forsteri*) during photopic and scotopic conditions. BL s^{-1}; body length units s^{-1} (fork length). Data points represent means and error bars are 95% CI. If the intervals overlap, treatments are statistically different

Both control and treatment groups under photopic and scotopic conditions were exposed to a stationary obstacle in order to quantify the role of the LLS in a school's ability to detect and adapt their group behaviour (avoidance). The obstacle was colourless and transparent (clear Plexiglas®) in an effort to reduce visual sensory input in the photopic groups. Results from a logistic regression (collision rate vs. number of exposures) showed a significant difference between control and treatment fish within both photopic and scotopic groups ($P < 0.001$; $P = 0.005$ respectively). There was a ~3-fold increase in the collision rates of schools of treatment fish without a fully functioning LLS with schools making contact with the obstacle ~80% of the time under photopic conditions. Under scotopic conditions collision rates increased ~2-fold (~65%) in comparison to control groups (Fig. 7).

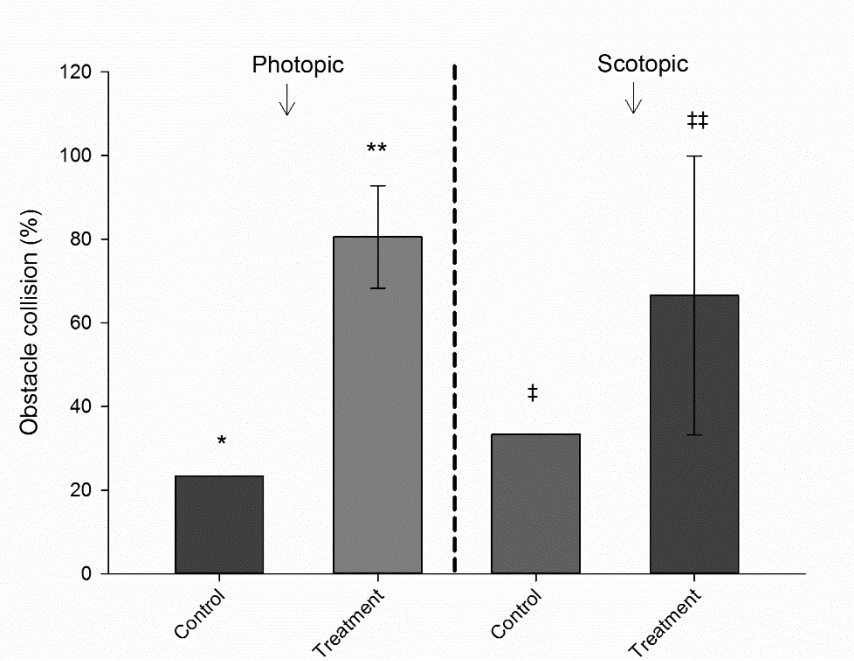

Figure 7 Obstacle collision rates for yellow-eyed mullet (*Aldrichetta forsteri*) schools with an inactivated lateral line system (treatment), expressed as a proportion (%) of the total number of passes around the tank. Significant differences within photopic and scotopic groups are marked with * or ‡ a significance accepted at $P \leq 0.05$.

7.5. Discussion

7.5.1. Neuromast morphology

Our morphological investigations showed trunk lateral line scales of yellow-eyed mullet each house a single oval shaped SN recessed in an independent longitudinal groove with the long axis of the SN situated in a rostrocaudal direction. Each contains hundreds of ciliary bundles located centrally in an oval area on the neuromast and each bundle contains a single kinocilium and several stereocilia (~10–20) of graduated length, polarised in opposing directions along the fish's rostrocaudal axis. Whilst much variety exists in SN morphology between fish species (e.g. shape, number of ciliary bundles and location on the SN, and number of stereocilia within bundles (Webb, 2011)), all neuromast ciliary bundles contain the same basic morphology

including a single kinocilium and several shorter stereocilia (Bleckmann & Zelick, 2009; Fischer et al., 2013), as supported by our results in yellow-eyed mullet.

Some species within the family Mugilidae show similar neuromast morphology and distribution as seen in the trunk LLS of flathead grey mullet (*M. cephalus*) which also possesses a single SN recessed in an independent longitudinal groove on every scale, totalling around 1100 (550 per side) (Ishida et al., 2015). Liu and Shen (1993) investigated distribution of neuromasts in 31 species of Mugilidae and found a total of ~1200 SNs (600 per side) constitute the LLS of yellow-eyed mullet, and are distributed on nearly all scales along 11–12 distinct lateral lines per side. Combining the total number of SNs (~1200) and hair cells/SN (~275) gives a remarkable total of around 330,000 innervated hair cells on the trunk of yellow-eyed mullet. The sensitivity of fish to various types of hydrodynamic stimuli is related to the type, number, distribution and orientation of neuromasts (Fischer et al., 2013). Our morphological investigations were confined to the trunk LLS and results showed that yellow-eyed mullet have developed a highly sensitive trunk SN mechanosensory system, with a distinct lack of trunk CNs, which raises the question of why? Perhaps as Appelbaum and Schemmel (1983) suggest, CN pores can become blocked with particulate matter and therefore the turbidity levels found commonly in estuarine habitats would render them less effective than SNs which project from the skin into the water (Kasumyan, 2003). This is an area for future research.

The functional units of the LLS (SNs and CNs) are morphologically different and perform separate functional tasks when detecting hydrodynamic stimuli due to differences in sensitivity (i.e. SNs for velocity and used for rheotaxis; CNs for acceleration and used for predator detection) (Engelmann et al., 2000; van Netten & McHenry, 2013). Yellow-eyed mullet schools live in highly dynamic coastal habitats subjected to constant changes in tidal flow, high tidal velocities and associated turbulence. It is therefore reasonable to assume that a highly developed superficial trunk LLS plays a bigger role in detecting changes in this environment

than could be achieved by canal neuromasts. However, Schmitz et al. (2014) in studies on four species of European cyprinids found no significant relationship between habitat use and distribution or orientation of either SNs or CNs. Given that yellow-eyed mullet are an obligate schooling fish, perhaps the answer lies more in group behaviour than habitat.

7.5.2. Schooling behaviour and object detection

School structure was altered in groups of yellow-eyed mullet with full use of vision (photopic) but without the use of a functioning LLS (i.e. reduced SA, NND, and swimming speed). Schools swam closer together and at a slower rate, which likely indicates that the disruption caused to sensory input created uncertainty amongst the group resulting in a packing down effect to increase safety. Reduced spacing between nearest neighbours after lateral line ablation was also found in other species, e.g. golden shiner, (*N. crysoleucas*) (Burgess & Shaw, 1981), and firehead tetra (*H. bleheri*) (Faucher et al., 2010).

Reduced variation (smaller confidence intervals) around the mean NND and SA under photopic conditions suggests that individuals exercised increased precision in their positioning within the school (i.e. tighter and more uniform inter-individual spacing and polarity). This maintenance of cohesive schooling behaviour indicates that there is heightened awareness of individual positioning and less disarray and/or random behaviour experienced by the group when the sensory modality of the LLS is lost and enhanced visual effort is required. We also suggest that a reduction in swimming speed (therefore reduced energetic expenditure) is an adaptive behaviour in yellow-eyed mullet to assist in increasing cohesiveness by focusing on spatial positioning (i.e. NND and SA). These results provide evidence for a prioritised multi-sensory (vision + LLS) approach being used by yellow-eyed mullet for optimisation of schooling behaviour. A sensory hierarchy is found in sharks that is determined by sub-modality availability (Gardiner et al., 2014) and our results are similar to seminal research by Pitcher et al. (1976) on saithe (*Pollachius virens*) who also found that vision is not vital in schooling

when the LLS is present. However, schooling is not possible with simultaneous and complete loss of both senses (Pitcher et al., 1976).

During the scotopic experiment, yellow-eyed mullet under reduced light conditions, and without the use of the LLS, increased their NND (as opposed to decreased NND in photopic groups, and similarly reduced swimming speed). These observations support the previously discussed visual effort hypothesis whereby loss of LLS function is compensated for by visual senses, but when light is limited these visual senses are unable to adequately maintain normal schooling behaviours. Our results support previous research findings that without the use of either of these sensory systems, group cohesion is decreased or lost (Faucher et al., 2010) and findings from feeding behaviour in muskellunge (*Esox masquinongy*) found that loss of the LLS negatively impacted foraging success (New et al., 2001), highlighting its importance to general fitness in fish. Azuma and Iwata (1994) found that maintenance of NND in coho salmon (*Oncorhynchus kisutch*), under varying light intensities, is primarily the function of vision, however, Cahn (1972) showed that in schooling mackerel tuna (*Euthynnus affinis*) detection of hydrodynamics by the LLS is also critical to maintaining NND. The lateral line system also plays a role in efficient locomotion as is supported by Yanase et al. (2014) who found that ablated trunk SNs negatively affected directional stability in yellowtail kingfish (*Seriola lalandi*). Given the obvious role of directional stability in maintaining school formation, it is plausible that yellow-eyed mullet decreased their swimming speed in both photopic and scotopic experiments to not only maintain locomotory efficiency, but also to retain a cohesive school structure.

The LLS also enables fish to detect and discriminate stationary objects in light or dark conditions due to currents that are generated from water flow passing around the object as was found in studies of Mexican blind cave fish (*Anoptichthys jordani*) (Von Campenhausen et al., 1981) and Sacramento splittail (*Pogonichthys macrolepidotus*) (Mussen & Cech Jr, 2013). In

still water under both photopic and scotopic light conditions, yellow-eyed mullet schools with ablated LLSs failed to detect and avoid a transparent stationary object significantly more often than control groups. Increased collisions in the photopic treatment group (i.e. non- visually impaired) highlights the role that this system plays in obstacle detection, which would be most valuable when environmental conditions reduce visibility (i.e. reduced visual ability in often turbid estuary environments). It was considered that a degree of flow would have been generated by the continuous swimming motion of the school (whirlpool effect), and would have contacted the object causing disturbed water that was detectable by the LLS in control groups under both light conditions. Windsor et al. (2010) found that in Mexican blind cave fish (*Astyanax fasciatus*) detection of the hydrodynamic stimulus generated when approaching a stationary object was carried out by neuromasts contained on the first 20% of the fish's body. This would explain why the obstacle was detectable to control groups of yellow-eyed mullet in a tank with no artificial flow generation. In the wild, and under reduced light and/or high turbidity (e.g. estuaries), it is likely that the large numbers of SNs present on the trunk of yellow-eyed mullet play an important role in obstacle detection and avoidance by fish schools.

Little is known about the neuroethology underpinning the relationship between the LLS and schooling behaviour, however our results clearly show the important contribution the LLS makes to yellow-eyed mullet schooling behaviour. Yellow-eyed mullet are an abundant inshore estuarine species faced with constant adjustments in schooling behaviour in response to changing biotic (i.e. predatory encounters) and abiotic factors such as tidal flow (currents and associated changes to water depth), obstacles (e.g. objects washed into estuaries on incoming tides) and the often high levels of turbidity resulting in reduced vision. Our findings improve the limited knowledge of yellow-eyed mullet biology and suggests the LLS plays an important role in managing the constant environmental fluxes found in estuarine ecosystems.

7.6. Ethics statement

All experiments were conducted in accordance with the University of Canterbury Animal Ethics Committee (Ref: 2015/02R). Methods were carried out in accordance with approved guidelines.

8 Foraging behaviours of teleosts at an estuarine feeding station

8.1. Abstract

Global demand for increased fisheries productivity traditionally led to the development of new/improved sustainable production methods, such as technological advances to fishing gear, sea-cage farming, and wild stock enhancement programmes. More recently, research has begun to focus on the potential use of inshore feeding stations to augment production via herding of wild fish. Inshore and estuarine coastal environments provide habitat for many aquatic species. Therefore, supplementary feeding is likely to attract multiple fish species. Currently, there is limited knowledge on the behavioural interactions or phenotypic composition of naturally formed fish schools whilst foraging at estuarine feeding stations. In this study, supplementary feed was discharged into an estuary via a land-based feeder. Species richness, size-assortiveness, interspecific relationships, and behavioural responses were assessed using acoustic (sonar) and video observations in non-predated mono- and mixed-species foraging groups, and in mono-species foraging when under avian predatory attack. Species attending the feeding station predominantly included yellow-eyed mullet (*Aldrichetta forsteri*), kahawai (*Arripis trutta*), and yellowtail kingfish (*Seriola lalandi*), while the presence of piscivorous birds (e.g. pied shag (*Phalacrocorax varius*) were also observed. Fish showed high temporal association rates to the feeding station, regardless of predator/competitor presence. A high level of size-assortment was found in schools. Latency time between aerial predator strikes and resumption of foraging in yellow-eyed mullet averaged ~4 s, and a larger distance was maintained (~three-fold) away from teleost competitors compared with aerial predators. Feeding hierarchies were observed with yellowtail kingfish foraging exclusively from the outlet pipe, whereas kahawai and yellow-eyed mullet foraged mutually. The aim of the research was to investigate the potential use of anthropogenic feeding stations for augmentation of inshore fisheries production by identifying specific foraging behaviours and predator-prey interactions.

8.2. Introduction

Global marine fish capture rates have risen annually since the 1950s from around 16 to nearly 83 million tonnes in 2014 (FAO, 2014). This correlates with increased global food demand, which looks set to continue with human population predicted to increase from the current ~7.3 billion to around 8.5 billion by 2030 (UNDESA, 2017). Commercial fishery capture production methods typically involve fishing vessels using trawl and purse seine nets to harvest large numbers of schooling fish (Prosser, 2015; Wardle, 1986). Operating costs are substantial and catches often include damaged, under-sized and non-target species (Halldorsson et al., 2012; Wilson et al., 2014). The development of new and improved fishing technologies and management practices is necessary in order to maximise sustainable production to meet global demands (Halldorsson et al., 2012).

Herding of wild fish schools has received increased research attention as an alternative method for sustainably optimising seafood production. This usually involves the use of acoustic cues to recruit naturally occurring wild fish to feeding stations for capture (Bjornsson, 2011; Zion & Barki, 2012). Compared with other production methods (e.g. fish farming), this is thought to be less detrimental to the environment (e.g. sedimentation effects from fish farms) whilst achieving cost reductions (e.g. lower overhead and maintenance costs) (Lindell et al., 2012). To date, herding potential has been investigated in very few species; however, successful feeding station recruitment was found in black sea bass (*Centropristis striata*) (Lindell et al., 2012), and a pilot study in Iceland showed sustained recruitment and tripled growth rates in free-ranging schools of Atlantic cod (*Gadus morhua*) (Bjornsson, 2011). A feasibility study comparing ranching to other production methods (e.g. sea-cage culture) found ranching to be the most profitable option (Halldorsson et al., 2012). However, research to date has only appeared to attract mono-species or monophyletic assemblages (i.e. gadoids).

Whether feeding stations operating in presently untested habitats (i.e. estuaries, or shallow coastal waters) attract a greater diversity of species is largely unknown. Inshore/estuarine habitats are complex tidal environments that are home to many fish species; therefore, estuarine-based supplementary feeding stations will potentially involve some level of multi-species interaction and predator-prey encounters. Such interactions may affect attendance rates (e.g. fish avoiding the feeding station), and therefore influence potential capture rates of target species. However, in light of the high importance of foraging success on an individual's growth and survival, the prospect of potential energy gains may contribute to adaptive behaviour and maintained foraging at the feeding station in the presence of apex predators, such as size-assortiveness in group formations.

Schooling behaviour (collective living) primarily improves individual fitness (Partridge, 1982), and fish show preference for group formation with phenotypically similar individuals. This preference, seen in both wild and laboratory conditions, frequently translates into fish electing to school with individuals of the same size (i.e. mass or body length), for example, three-spined stickleback (*Gasterosteus aculeatus*) and European minnow (*Phoxinus phoxinus*) (Hoare et al., 2000; Ranta et al., 1992; Ward & Krause, 2001). This phenomenon is also seen in multi-species schools of golden shiner (*Notemigonus crysoleucas*) and banded killifish (*Fundulus diaphanous*) (Krause et al., 1996a). There is also evidence that the level of size-assortment increases with increased predation risk, as shown in bluntnose minnows (*Pimephales notatus*) and Trinidadian guppy (*Poecilia reticulata*) (Croft et al., 2009; Theodorakis, 1989), supporting the suggestion that predation asserts a selection pressure that drives phenotypic assortment in fish. In addition to size-assortiveness fish have also developed an array of predator avoidance behaviours that result in a change of group structure (Fuiman & Magurran, 1994). These include: fountain effects, flash expansions and vacuoles, which are employed once a predation threat has been identified (Hall et al., 1986; Lett et al., 2014; Partridge, 1982). As a result,

separation distance is momentarily increased between predator and prey causing disruption to the cohesive structure of the school before collective behaviour and structure resumes.

Investigations into the potential for establishing feeding stations to augment fisheries production are rare. Therefore, knowledge of foraging hierarchies, seasonal variation in feeding station fidelity, phenotypic assortment and behavioural response to predators remain largely unknown. In addition to examining how inter-specific competitive behaviours shape community structure at feeding stations, observations of behavioural traits will also help elucidate other aspects of shared habitat. The current study aimed to quantify the behavioural characteristics of multi-species fish assemblages at an estuarine-based feeding station. To the authors' knowledge this is the first study of its kind to address the behavioural interactions associated with supplementary feeding used for recruitment, on-growth and subsequent harvest of wild free-ranging inshore Australasian fish species. These findings are likely to contribute to improved fisheries capture productivity in support of the growing global demand for seafood.

8.3. Materials and methods

8.3.1. Experimental setup

A feeding station was established and operated in the Nelson Haven estuary, New Zealand (41.254° S, 173.279°E) (Fig. 1).

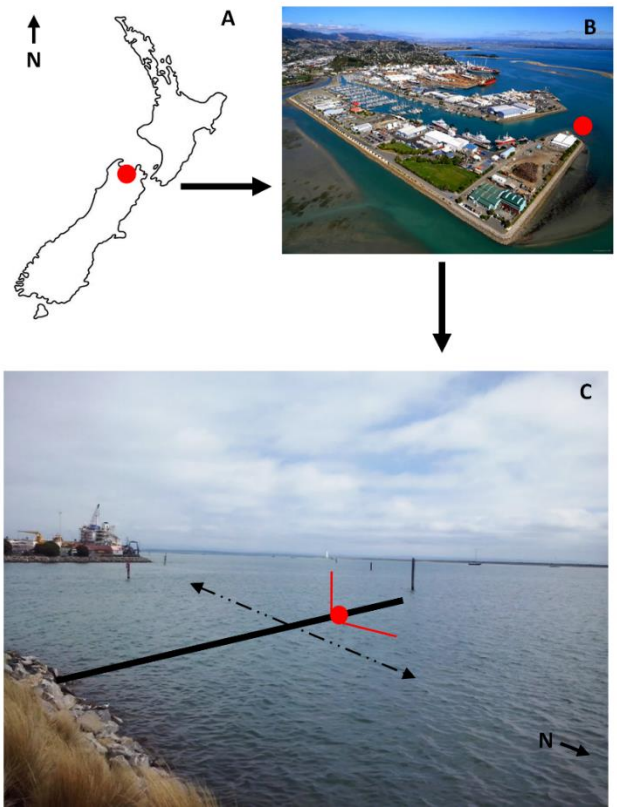

Figure 1 Approximate sub-surface positioning and field of view for ARIS sonar (C) (red circle and lines) in relation to feeding station outlet pipe (solid black line) in the Nelson Haven, New Zealand (A–B). Tidal flow direction indicated by dotted arrow. Photo credits: (A–B) Google images, (C) K. Middlemiss.

The device consisted of a land based 600 L silo from which frozen feed (~18 mm particle size) was periodically flushed into the estuary along a subterranean 50 mm diameter pipe to the feed outlet (Fig. 1C), which was exposed and located at mean low water springs (MLWS) (0.4 m charted depth). The feeder was in operation during daylight hours, under suitable tidal conditions (i.e. above 2 m charted water depth, mean high water springs (MHWS) = 4.2 m). This ensured a sufficient column of water was available for fish to forage in.

Acoustic observations were performed using an imaging sonar (ARIS 3000, Sound metrics Inc., WA, USA). The sonar was positioned at a tangent to the tidal flow across the feed station, 6–7 m from the feed outlet (Fig. 1C). Recordings were captured at a frequency of 1.8 MHz with a frame rate of 3.8 frames per second (FPS) at a resolution of 3 mm. During the recording

period the sonar was programmed to sweep across the feeding station at a rate of 0.5° s^{-1}, with the feed outlet centred in the swath, providing imagery across a 110 m^2 survey area. Time-lapse video and photographs were captured with a GoPro® Hero3 (Woodman Labs, CA, USA) (1080p, 30fps, wide field of view) and intervalometer board (Camdo Solutions, WI, USA), set to 30 sec video grab every 20 min.

8.3.2. Species richness

Species richness data (% time that each species was present in a series of 10 observations), was measured using GoPro® camera images, whilst fish actively foraged from the feeding station, between the observation period of November 2014 to June 2015. It should be noted that data for the month of December is missing from species richness analysis due to technical issues with equipment. Individual species were recorded as present or absent on two separate days each month, on five separate occasions (20 min apart), and over an approximate 1–2 h period, during feeder operation on each day (i.e. n=10 total observations per month).

8.3.3. Size-assortiveness

Size assortiveness was quantified in schools foraging at the feeding station during three mutually exclusive behavioural states. A total of nine schools consisting of n=3 each during: (1) mono-species foraging (i.e. yellow-eyed mullet (*A. forsteri*) exclusively foraging), (2) mixed-species foraging (i.e. kahawai (*A. trutta*) were present with yellow-eyed mullet), and (3) mono-species foraging with an avian predator present (i.e. yellow-eyed mullet and pied shag). Each school was defined as fish attending the feeding station on separate days over consecutive 3–4 day observation periods. Schools in October 2014 consisted of mono-species and mono-species foraging with an avian predator, while mixed-species foraging was recorded in January 2015. Using ARIScope® software, sonar observations were analysed and total fish length (TL) calculated. On each of the nine sample days, 30 individual fish were randomly sub-sampled

from n=3 sonar frames (10 fish per frame), giving a total of n=30 TL measurements for each school. Due to limitations with the sonar field of view (FOV), and the size of fish aggregations, it was not possible to simultaneously visualise all fish within the school, therefore sub-sampling gave a representation of the level of phenotypic (body size) variability for the whole school. The following sampling method was adopted to reduce the likelihood of repeat measures of individuals and/or selection bias of individuals within each school. A quadrat sampling system was used to randomly select 10 fish by overlaying the ARIScope® range/bearing grid software function on to each sonar frame (Fig. 2A). A total of 24 consecutive quadrats was selected, at a distance >3 m from the sonar (to limit the effect on length accuracy caused by crosstalk between sonar beams closer than 3 m (Middlemiss, 2017)). Quadrats were assigned a unique identifier (i.e. numbered 1–24, left to right, top to bottom). A random number generator was used (R v. 0.97.551) to select 10 non-repeated quadrat numbers, and one fish from each quadrat was selected for measurement. Where >1 fish was present in each quadrat, the fish positioned in the lowest part of the quadrat was selected, and if nil fish were present new quadrat numbers were generated until a total of 10 fish were identified. Each measured fish was tracked through successive frames until presenting in an 's' shape to the sonar; therefore, the head and tail were clearly visible, ensuring accuracy of length measurements. To reduce the possibility of repeat measures of the same fish, each frame was separated by >30 s intervals (i.e. >100 frames).

8.3.4. Anti-predator behavioural responses

8.3.4.1. Latency

During previously described sonar observations of fish attending the feeding station, ad hoc instances of predator-prey interactions were observed and recorded. Using the sonar imagery, latency time (min) between avian predator attack (pied shag) and resumption of foraging behaviour, was analysed with ARIScope® software. Time was calculated from the number of frames between start and finish measurements, divided by the frame rate (3.8 s), for n=16 bird

strikes. Identification of the avian predator species was confirmed from GoPro® footage taken simultaneously with sonar footage. Piscean attack latency could not be measured due to nil instances of teleost-teleost predation being observed.

8.3.4.2. Separation distance

During predation or competition threat from avian (i.e. pied shag) or teleost (based on size, i.e. kahawai and yellowtail kingfish (*S. lalandi*)) species respectively, the vacuole of space created (resulting from avoidance responses (i.e. flash expansion), was described by calculating the mean distance (mm) between the threat stimulus and the nearest fish (n=10 each avian and teleost events). Observations were made from sonar footage generated over the period 8–10 of January 2015 (teleost), and 13–14 October 2014 (avian). Using ARIScope® software, the mean distance between teleost competitor and nearest fish in the school was calculated from four directions: anterior, posterior, left lateral, and right lateral, using the focal fish's head, tail, and lateral midpoints respectively (Fig. 2B). For school response to avian predation, the mean distance was calculated from four measurements from the centre of the vacuole (based on sonar x and y coordinates for the bubble trail of the diving bird), standardised at 0, 90, 180, and 270° angles. To enable measurement in all four spatial directions frames were only selected for measurement when the vacuole was completely surrounded by fish (i.e. 360° encirclement) as shown in Figure 2B. Measurements were further standardised by selecting the measurement frame at which the fish group had reached maximum expansion away from the predation threat.

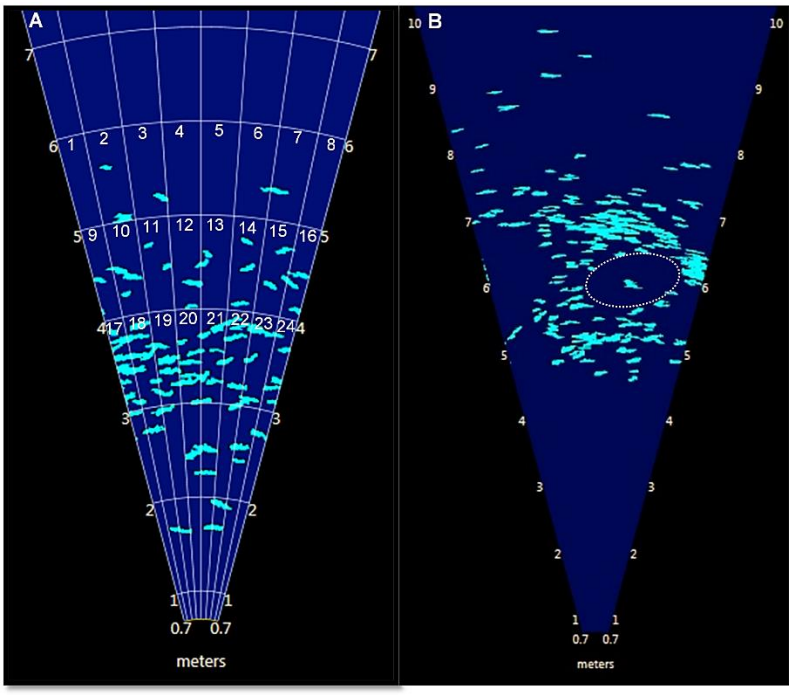

Figure 2 Sonar image depicting (A) a fish assemblage used to calculate fish length (TL, total length) at an anthropogenic feeding station, showing a quadrat measuring system numbered 1-24, and (B) vacuole surrounding a predator (i.e. teleost) marked in the centre of the dotted circle.

8.3.5. Statistical analysis

Analysis was carried out using SigmaPlot v.12.5, Minitab 16 (species richness data only), and GenStat (fish length homogeneity of variance). Species richness data were analysed using a binary logistic regression. Size assortiveness data were square root transformed and analysed using 2-way ANOVA, using a Tukey test used for multiple comparisons. Homogeneity of fish lengths within schools was tested for equal variance using the Levene's test (GenStat), and 1-way ANOVA for comparison of mean school sizes (Tukey test). Anti-predator evasion behaviour was analysed using 2-way ANOVA (factors predator/competitor type and observation frequency) and Tukey test for multiple comparisons. Data are represented as the mean ± 95% CI, unless otherwise stated, and significance was accepted at $P \leq 0.05$.

8.4. Results

8.4.1. Species richness and temporal feeding station attendance

Monthly variations in species richness during feeding station operation showed that yellow-eyed mullet (*A. forsteri*) were present on all occasions, except in April (Fig. 3). Species richness increased in February when yellow-eyed mullet, kahawai, and to a lesser extent spotted wrasse (*Notolabrus celidotus*) were observed foraging mutually. Kahawai were present in January and February and appeared less frequently between March and May, with only one occurrence being recorded in May. Spotted wrasse were only seen foraging in February and were present in 10% of observations that month. Yellowtail kingfish were present 50% of the time during March and were the only species observed at the feeder in April, when yellowtail kingfish foraged directly from the feed pipe to the exclusion of all other species. Notably over this period yellow-eyed mullet were present in the general area despite not being seen foraging at the feed pipe in video recordings during the feeder's operation when in the presence of yellowtail kingfish.

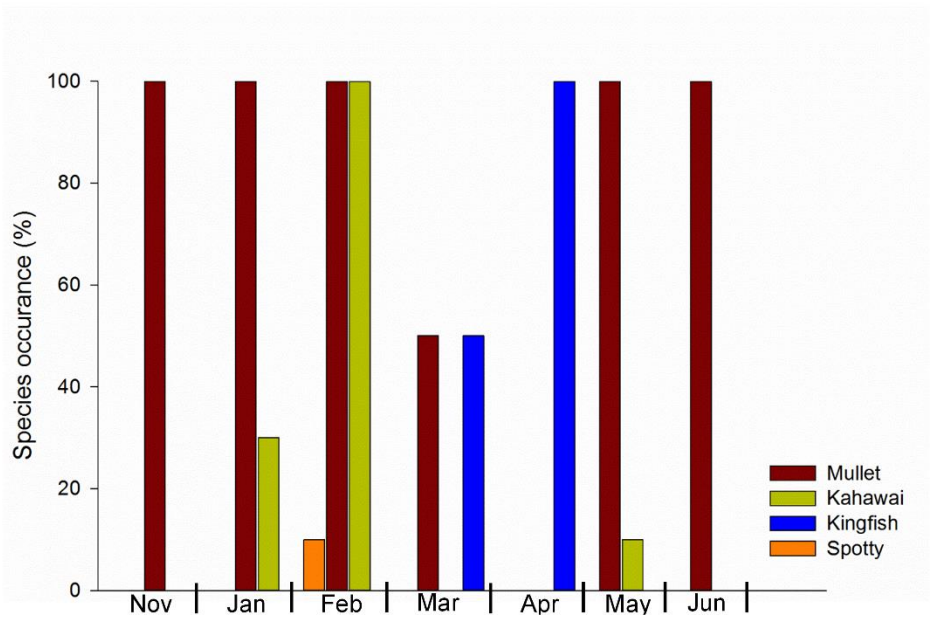

Figure 3 Species richness whilst foraging at a Nelson Haven estuary based anthropogenic feeding station between November 2014 and June 2015.

8.4.2. Qualitative foraging behaviour

Sonar and video observations showed the mode of feed consumption for fish schools presented with a point source of food was typically positively rheotaxic with schools aligned directionally towards the food source. Although there appeared to be little net spatial movement of the school in its entirety, the swimming speeds of the fish within the school were slightly greater than water current speeds, causing a steady but gradual movement forward to the feed outlet. Individuals momentarily positioned themselves directly over the feed outlet pipe and then successive individuals shuffled into positions of direct food delivery perhaps as a result of competition, but also likely due to the high density of individuals around the feeder outlet. After a brief period of positioning themselves at the feed outlet, fish either fell back within the mass of the school, or separated from the front moving laterally down the school's length before re-joining the group in positions further to the rear, analogous to the O turn manoeuvres described in (Domenici et al., 2002). When both kahawai and yellow-eyed mullet were present at the feeding station, foraging behaviours were broadly similar. Differences in species-specific

behaviour were observed in differing tidal flow conditions as kahawai appeared dominant in periods of high flow displaying more vigorous propulsion towards the feed source, likely indicating a high level of athleticism and foraging dominance in this species. Whilst individual species foraging at the feeding station could be identified from video footage, it was not possible to quantify the relative numbers or biomass of each species present using this method. Interestingly, during feeding station foraging, yellowtail kingfish, apex predators, excluded other species from direct access to the food outlet pipe, but did not display predatory behaviour towards schools of yellow-eyed mullet and/or kahawai. Therefore, feeding station interactions between these species were interpreted as more competitive than predator-prey by nature.

8.4.3. Size-assortiveness

8.4.3.1. Mono-species foraging

Mean body length of yellow-eyed mullet in schools one, two and three (R_1, R_2, and R_3) whilst foraging in the absence of other species at the feeding station were: R_1 181.6 ± 9.9 mm, R_2 247.0 ± 9.0 mm, and R_3 241.5 ± 8.2 mm (Fig. 4). Whilst the mean body length of fish was significantly smaller in R_1 than in R_2 and R_3 ($P < 0.001$), analysis of length variance (Levene's test of homogeneity) between all three schools showed a high level of size assortment within each as evidenced by the similarity of quantile-quantile (q-q) plot slopes.

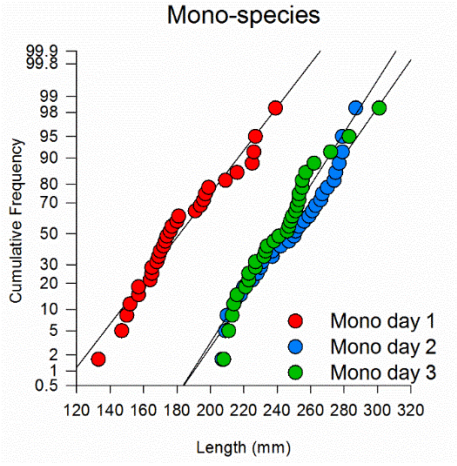

Figure 4 Individual fish lengths (total length) within mono-species schools of n=30 yellow-eyed mullet (*Aldrichetta forsteri*). Data points represent the normalised cumulative sums of the residuals with probability axes.

8.4.3.2. Mixed-species foraging

Analysis of the mean length of mixed-species schools (yellow-eyed mullet and kahawai) during feeding station foraging were: R_1 237.9 ± 6.5 mm, R_2 241 ± 8.9 mm, and R_3 236.1 ± 12.9 mm (Fig. 5). There was no significant difference in the mean body length of fish within each school (P = 0.764); however, there was slightly less homogeneity of variance lengths within R_3, which ranged between 184 and 322 mm compared with R_1 209–268 mm and R_2 205–312 mm.

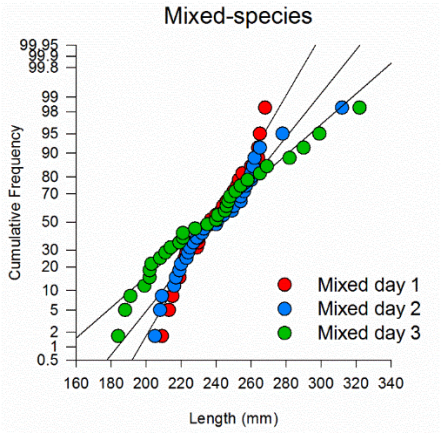

Figure 5 Individual fish lengths (total length) within mixed-species schools of n=30 yellow-eyed mullet (*Aldrichetta forsteri*) and kahawai (*Arripis trutta*) foraging at a feeding station. Data points represents the normalised cumulative sums of the residuals with probability axes.

8.4.3.3. Mono-species foraging with avian predator present

Schools of yellow-eyed mullet foraging at a feeding station in the presence of an avian predator (pied shag) had significantly different mean body lengths: R_1 243.4 ± 15.5 mm, R_2 184.2 ± 9.5 mm, and R_3 201.5 ± 9.9 mm (R_1 vs R_2 and R_3 $P < 0.001$ respectively, R_2 vs R_3 $P = 0.023$) (Fig. 6). Further analysis within each school showed the range of sizes also varied between schools with more variation in R_1 (173–332 mm) than in R_2 (135–226 mm), and R_3 (161–251 mm).

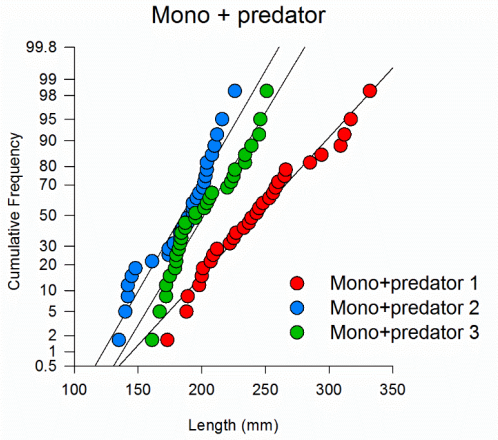

Figure 6 Individual fish lengths (total length) within mono-species schools of yellow-eyed mullet (*Aldrichetta forsteri*) foraging at a feeding station with an avian predator present. Data points represent the normalised cumulative sums of the residuals with probability axes.

8.4.3.4. Comparative analysis between all schools and behavioural states

When comparing all schools irrespective of behavioural state (i.e. mono-species, mixed-species, and mono-species + predator, Figs. 4–6) most (8 out of 9) schools had similar variation in lengths, with one of the mono-species + predator schools having a wider range of lengths (Levene's test, $P = 0.001$), size range being a measure of size-assortiveness (Fig. 7). Size distributions were normal; however, there was heterogeneity between schools in terms of mean length and size-assortiveness/spread of lengths (based on nested ANOVAs and Levene's tests), but no clear differences between the types of school in either mean length or size-assortiveness.

Interestingly, of the 270 fish measured in total only six had a body length ≥300 mm and five ≤150 mm.

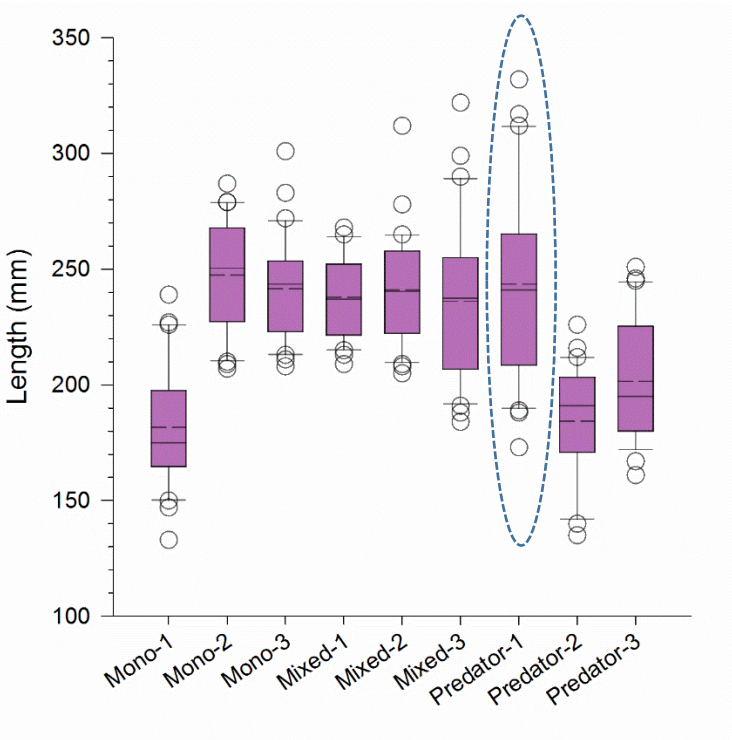

Figure 7 Body lengths of n=30 yellow-eyed mullet (*Aldrichetta forsteri*) foraging in mono-species (mono 1-3), mixed-species (mixed 1–3), and mono-species + predator (predator 1–3) schools. Data represented by box plots showing median (solid line), and mean (dashed line) lengths. Upper/lower quartiles around the median lengths (coloured box) represent 50% of fish within each school and whiskers extend to longest and shortest lengths, excluding outliers. Dotted circle represents the group with the highest size variance.

8.4.4. Anti-predator behaviour

8.4.4.1. Latency between bird strike and feed resumption

Yellow-eyed mullet predation threat from an aerial bird strike (pied shag) resulted in a range of 6.3 s (1.8 s to 8.1 s) latency between the time the school dispersed (flash expansion), then re-grouped to prior schooling and feeding behaviour (Fig. 8). Analysis showed the mean time response was 3.85 ± CI 0.96 s and 75% of aerial attacks (n=16) resulted in fish resuming cohesive schooling behaviour in ≤ 4.47 s.

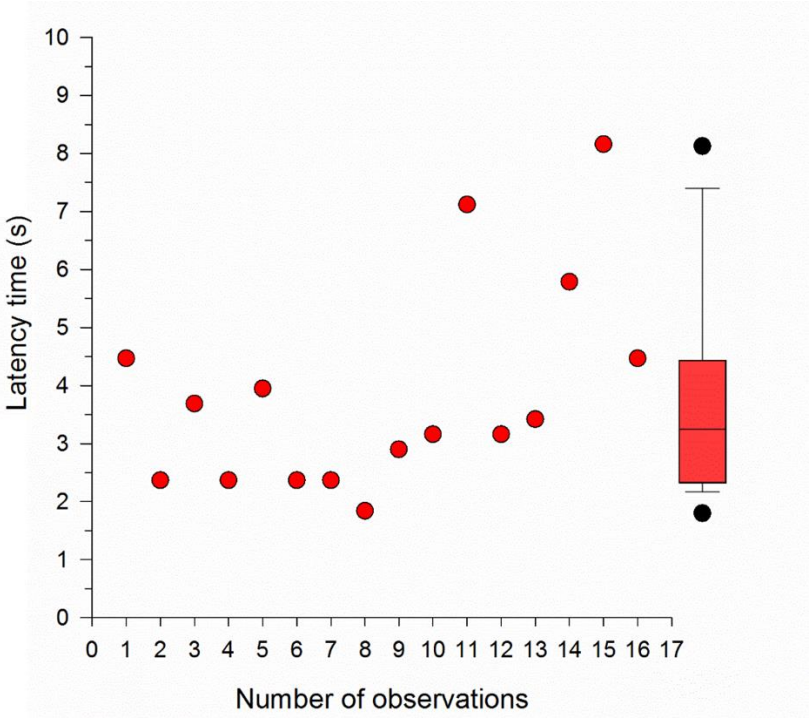

Figure 8 Latency time (s) in mono-species groups of yellow-eyed mullet (*Aldrichetta forsteri*), during avian predation (pied shag). Data points represent individual aerial strikes.

8.4.4.2. Evasion/avoidance behaviour

Schools of yellow-eyed mullet displayed flash expansion behaviour in response to predation/competition from avian and teleosts, forming a vacuole, as described in Figure 2B. Analysis showed a significantly larger mean separation distance (almost three-fold) between fish schools in the presence of teleost competitors (403.6 ± CI 55.3 mm) than when avian predators were present (114.8 ± CI 19.9 mm) ($P < 0.001$) (Fig. 9).

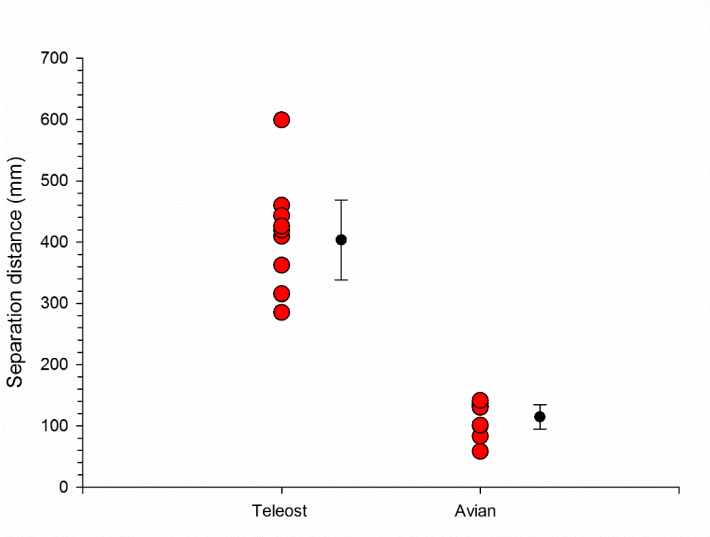

Figure 9 Mean separation distance between yellow-eyed mullet (*Aldrichetta forsteri*), during teleost and avian competition/predation. Data points represent individual responses. Error bars are 95% CI and significance was accepted at $P \leq 0.05$.

8.5. Discussion

Commercial fisheries continue to investigate the development of novel and sustainable fish recruitment and capture methods (e.g. wild stock enhancement programmes) (Bartley & Bell, 2008; Zion & Barki, 2012). Recent research places a growing focus on the potential for increased capture production through herding of wild, free-ranging fish with the use of supplementary feeding stations (Bjornsson, 2011; Zion & Barki, 2012). Studies have included feeding station fidelity, growth rates and migratory behaviour of Atlantic cod (*Gadus morhua*) (Bjornsson, 2002; Bjornsson et al., 2010), and the effects of social behaviour on single species fish aggregations (tunas (Scombridae) Robert et al., 2013). However, the current study aimed to address the considerable lack of knowledge on species recruitment, temporal attendance, interspecific behaviours and size-structuring associated with free-ranging mixed-species fish assemblages, which might affect foraging abundance at inshore feeding stations.

8.5.1. Species richness and feeding behaviour

Inshore ecosystems support many fish species (both permanent and transient), each occupying different food web trophic levels (e.g. predator/prey), and often competing for the same resources (e.g. nutrition and breeding grounds) (Elliott & Hemingway, 2008). The Nelson Haven is an estuary around 1300 ha in size, providing important feeding and nursery habitat for several inshore fish species, including snapper (*Chrysophrys auratus*), kahawai and yellow-eyed mullet (Gillespie, 2008), with Mugilidae suggested as a keystone species in many estuarine ecosystems (Crosetti & Blaber, 2016). Between November 2014 and June 2015 three teleost species were predominantly associated with the feeding station. Both kahawai and yellowtail kingfish were observed feeding periodically during the summer months sampled, with their absence during some months consistent with known seasonal migratory behaviour (Griggs et al., 1998; Walsh et al., 2003), whereas yellow-eyed mullet were present during all months sampled in summer, spring and autumn. A notable absence during all observation periods was juvenile snapper, which are believed to utilise eelgrass (*Zostera muelleri*) nursery habitat, including within the Nelson Haven (Cawthron, 2009; Parsons et al., 2016; Parsons et al., 2014). It is conceivable that snapper were present in the estuary but not captured on sonar images taken over the observation period indicating that snapper do not forage with other species and are, therefore, not suitable as target species for feeding station recruitment. In terms of capture production, yellow-eyed mullet are not currently a high value commercial species in New Zealand (<50 t annually) (Morrison et al., 2014); therefore, their high fidelity rates at the feeding station suggest potential for increasing their commercial viability.

Yellowtail kingfish, an apex predator, the diet of which includes yellow-eyed mullet (Walsh et al., 2003), competed for and foraged to the exclusion of other species. However, yellow-eyed mullet and kahawai were observed feeding mutually, consistent with tank-based studies on multi-species feeding behaviour in snapper, kahawai and yellow-eyed mullet (Middlemiss et

al., unpublished). This highlights distinct trophic level feeding behaviours, which are most apparent between large bodied piscivores (yellowtail kingfish) and more generalist smaller bodied predators and omnivores (kahawai and yellow-eyed mullet). This is of particular importance when considering species composition and target species for recruitment and capture at feeding stations (e.g. target species could be excluded from foraging by higher trophic level non-target predator species). Deudero (2001) suggested the development of a form of 'dietary segregation' as a possible solution to prevent foraging competition, a concept also discussed in (Hall et al., 1990).

Tidal flow also affected foraging behaviour, with a dominance hierarchy seen in kahawai over yellow-eyed mullet during periods of high tidal current speeds presumably, due to the energy expenditure needed to maintain station (rheotaxis) at the feeding station. This is consistent with other studies on the effects of tidal flow rates in estuarine species, which identified change to community structure and behaviour of fish under differing tidal conditions (Castellanos-Galindo & Krumme, 2015; Rieucau et al., 2015b). Future research investigating circadian feeding rhythms, food and feeding periodicity preferences in target species, species-specific optimal rheotaxic flow rates, and potential impacts of adaptive behaviour on estuarine ecology, including breeding migratory patterns, are required to identify feeding regimes suited to species of interest and to optimise production potential.

8.5.2. Size-assortiveness and mixed species interactions

Predation is a selection pressure contributing to size-assortment in fish groups (Croft et al., 2009; Theodorakis, 1989). Results showed small variation in mean size of fish between schools (predominantly yellow-eyed mullet); however, a high level of size-assortment (characterised as homogeneity of length within a school), was present regardless of competition for food or predation risk. This supports previous findings of phenotypic assortment in tank-based studies

of yellow-eyed mullet (Middlemiss et al., 2017c). One explanation for this is that fish of differing phenotypic characteristics are conspicuous within a group of dissimilar fish (known as the oddity effect), whereby predator attention is drawn towards such individuals and this may result in increased predation risk (Landeau & Terborgh, 1986; Ranta et al., 1994). Supporting this theory, guppy (*P. reticulata*) showed during a choice test a preference to shoal with similar sized fish (Jones et al., 2010). These findings suggest that yellow-eyed mullet, which were abundant at the feeding station, school in size-assorted cohorts based on a narrow range of acceptable phenotypic size variation. Our research suggests body size is a strong driver of phenotypic assortment in wild populations of yellow-eyed mullet.

Phenotypic assortment within fish groups can be further categorised by species, and formation of mixed-species fish groups is not uncommon among teleosts (Krause et al., 1996b), nor is mixed-species foraging (Lukoschek & McCormick, 2002). Indeed, yellow-eyed mullet have been shown to form schools with kahawai in tank-based studies and display no evidence of inter-specific aggression (Middlemiss et al., 2017d). The latter is of particular interest, given that kahawai are known predators of yellow-eyed mullet; however, it was assumed that similar morphometrics (i.e. body size) and food abundance prevented any aggressive behaviour, and perhaps promoted mixed-schooling behaviour. Interestingly, Middlemiss et al. (2017d) found that kahawai outcompeted yellow-eyed mullet during foraging; however, whilst food consumption rates were not measured at the feeding station, observations appeared to show similar levels of foraging success between these two species in the current study. Potentially, differences in the biomass of each species attending the feed station may have accounted for the apparent foraging success of the different species (not measured), whereby numerous yellow-eyed mullet could outcompete the much smaller numbers of kahawai present. Of particular note is that the range of size-assortment in schools foraging at the feeding station predominantly did not include fish smaller than 150 mm or larger than 300 mm. This may

indicate that smaller juvenile fish were outcompeted, due to body size, and that fewer larger fish are present in the estuary perhaps due to migratory patterns in larger adult fish resulting in movement between estuarine and inshore habitat.

8.5.3. Anti-predator behaviour

Evasive manoeuvres, such as flash expansions and vacuoles seen in schools responding to both avian and teleost predation/competition, are common among species (Partridge, 1982), and are typically short-lived responses (Marras & Domenici, 2013). The factors affecting types and latency of avoidance responses have been well studied in many species (reviewed in Domenici, 2010; Lima & Dill, 1990); however, to the authors' knowledge, latency time between reaction and resumption of foraging behaviour at a feeding station is unknown. Yellow-eyed mullet mean latency between initial reaction and return to foraging behaviour was around 3.9 s. Given that avoidance tactics caused disruption to school structure (i.e. fish became separated during flash expansion) it is not surprising that yellow-eyed mullet quickly regained group formation after an avian predation event, because this allowed them to quickly switch from anti-predator to foraging behaviour. Given that schooling behaviour decreases the risk of predation (Partridge, 1982), predation selection pressures would favour groups of fish that regain coordinated behaviours as soon as possible – a theory clearly supported by the immediate resumption of schooling (and feeding) behaviours in yellow-eyed mullet.

Reaction distance between predator and prey depends on factors including the perceived level of predation risk (Domenici, 2010). Tank-based studies on fathead minnows (*Pimephales promelas*), exposed to predation attacks from four separate teleost predators, displayed reaction distances of between 61 mm and 106 mm (Webb, 1982). However, and of particular interest, the maximum distances obtained in groups of yellow-eyed mullet during avoidance responses differed significantly between teleost competitor and avian predation threats. The almost

three-fold distance increase (mean 404 mm) generated by teleost competition, compared with 115 mm during aerial predation, suggests either prey had more time to identify and react to the teleost competitor than for the avian predator, or that a relationship exists between latency time and the length of time a predator is present. It could also indicate that the perceived risk to fish from the teleost predator is greater than in the avian predator. Therefore, separation distance may be relative to the likelihood of continued interaction with the predator species. Jointly, results on latency time to foraging resumption and reaction distances in yellow-eyed mullet clearly showed that feeding behaviour, in terms of feeding station attendance, was not permanently altered in groups during avian predation, suggesting a strong motive to feed from the feed station in this species.

8.6. Summary

The use of anthropogenic feeding stations to increase commercial capture fishery production is a growing area of research. However, little is known about the relationships and behavioural interactions between multi-species whilst sharing the same food resource. Inshore habitats support commercially important fish species that occupy differing trophic levels in the food web. Kahawai, yellow-eyed mullet and yellowtail kingfish dominated the feeding station in the current study. The latter, an apex predator, foraged to the exclusion of the other two species. Yellow-eyed mullet showed high temporal association to the feeder, displayed schooling behaviour and foraged mutually with kahawai. A high degree of size-assortiveness was shown, species quickly resumed schooling/feeding behaviour after avoidance responses, and maintained greater distances away from teleost than avian predators/competitors. This research adds further support to the existing body of knowledge describing the potential of feeding stations to augment inshore fisheries production through recruitment and on growth of commercially valuable species.

8.7. Ethics statement

The installation and operation of this feed station was performed in accordance with regulatory resource consent requirements approved by the Nelson City Council, New Zealand (RM145057).

9 General discussion

9.1 SUMMARY OF RESULTS

This thesis investigated two questions relating to collective behaviour in teleost species:

1. What sensory and behavioural factors contribute to group formation and interactions in fish species?
2. How do foraging and anti-predator behavioural traits associated with sympatric species affect individual fitness at a feeding station?

The graceful and seemingly effortless co-ordination displayed in teleost schooling behaviour belies the many behavioural traits and interaction rules involved in this phenomenon. These include the maintenance of optimal fish separation distances, polarity, and swimming velocity (Parrish et al., 2002). Interaction rules governing these traits are strongly influenced by factors such as (1) species composition, (2) group size, and (3) phenotypic assortment.

This thesis aimed to identify behavioural traits associated with collective behaviour in three sympatric New Zealand species; yellow-eyed mullet (*Aldrichetta forsteri*), kahawai (*Arripis trutta*), and snapper (*Chrysophrys auratus*), whilst considering the above three factors, during conditions of predation and foraging. The thesis also sought to investigate the role of key sensory systems in schooling behaviour, and then, combining all the findings from tank-based studies, to compare results to behavioural interactions in wild populations of these sympatric estuarine species. Collective behaviour described here has been divided into two swimming categories: schooling and shoaling behaviour. Schooling is defined as synchronised and polarised swimming and shoaling as a social group displaying no synchronisation or polarisation (Pitcher, 1983).

We investigated mixed-species groups of yellow-eyed mullet, kahawai, and snapper, and found that upon introduction of all three species in a single tank, yellow-eyed mullet and kahawai formed and maintained a single group displaying cohesive schooling behaviour (Chapter 3). Kahawai are known predators of yellow-eyed mullet (Baker, 1971); however, considering the

similar morphometrics of both species used in this experiment, and the propensity for natural populations of both species to engage in schooling behaviour (Morrison et al., 2014), this was interesting, but unsurprising. Had a size differentiation existed between the two species this may have resulted in interspecific aggressive behaviour. Snapper, however, displayed shoaling behaviour within a distinctly separate sub-group and preferred to occupy a position in the water column beneath the mixed-species school of yellow-eyed mullet and kahawai. Given the continuous rotational swimming behaviour displayed by the mixed-species school, it is likely that the shoaling behaviour displayed by snapper forced them to segregate themselves to avoid collision with the yellow-eyed mullet/kahawai group.

During foraging, kahawai were the most dominant of the three species in terms of both food consumption and lead positioning within the yellow-eyed mullet/kahawai school (Chapter 3). Whilst there was no clear sub-grouping of the two species within the school (i.e. all kahawai did not swim close together), the kahawai quickly broke from the school in an aggressive attempt to outcompete the other species and secure the food source. School structure, in response to aerial and diving predation threats, remained constant between both these behavioural states in the yellow-eyed mullet/kahawai group, but was lost during feeding with increased separation distances, angles and swimming velocity. This highlights the fact that during conditions where individual survival is threatened, fish seek the benefits of reduced predation risk associated with group living and in doing so apply the collective interaction rules necessary to maintain group cohesion. However, this was of less importance during foraging, likely because individuals in schools were competing with each other and, therefore, positioned themselves (i.e. spread out) to increase individual foraging success. Similar behaviour was also seen in snapper.

Interestingly, differences in swimming velocity were evidenced in individual species within the yellow-eyed mullet/kahawai group. This would suggest that although mixed-species groups

reach consensus around the interaction rules required to maintain group cohesion, a level of discordance exits within these groups that is perhaps associated with nuances between individual species physiological abilities. The fact that two species, having never schooled together previously, and given there is no known literature suggesting they do so in the wild, can instantly form and maintain a cohesive school, suggests convergent or ancestral evolution of behavioural traits is involved in schooling. All intraspecific behaviours and interspecific interactions found in this thesis likely exist in wild populations; however, confirmation of this would require future research, although is partly addressed by findings in Chapter 8. Importantly, results have highlighted the complex nature of feeding and predatory behaviours in fish species utilising shared habitat which results in interactions between associated species; yellow-eyed mullet, kahawai, and snapper.

To identify the role that local and global properties play in group structure, we also investigated the effects of group size on spatial patterns associated with group formation in yellow-eyed mullet during foraging and predation conditions (Chapter 4). Results showed that the size of the group had a direct relationship with the level of isotropy related to inter-individual spacing in fish, with more free-space around individuals in larger groups. In addition, the vacuole of free-space surrounding individual fish was spherical with an even distribution. Schools formed an oblong/spheroid with a length, breadth and height ration of 5:2:1. Our results highlight that rules governing behavioural traits fundamental to maintenance of group structure are intrinsically associated with the numbers of fish in the group, as well as the interactions between nearest neighbour fish. These findings are important because they challenge the traditional understanding that it is either local *or* global properties that are the key driver of cohesive behaviour in small fish groups (Rieucau et al., 2015a; Tunstrom et al., 2013). Therefore, a more integrative approach to future research should be taken to elucidate the interactive effect of various mechanisms governing group structure.

A common phenomenon in group formation is that fish tend to group with phenotypically similar individuals to reduce the likelihood of predation by minimising phenotypic oddity, therefore improving individual fitness (Landeau & Terborgh, 1986). In particular, size-assortment is believed to be a key driver in group formation (Ranta et al., 1994; Theodorakis, 1989), and the current study identified interactions in mono-species groups of different sized fish (small and large, yellow-eyed mullet and snapper), and quantified the effects on group structure during foraging and predation conditions (Chapter 5). Results showed that, given the choice, yellow-eyed mullet maintained size-assorted schools and conversely, snapper formed a single shoal. Group structures differed and were dependent on species, size-assortment, and behavioural conditions. This suggests that species-specific swimming behaviours (i.e. schooling vs shoaling) are a key driver in size-assortiveness and interaction rules governing group formation and structure. Furthermore, it is likely that the high level of size-assortiveness found in yellow-eyed mullet also exists in wild populations and, therefore, the total number of fish groups present in these spatially limited environments is likely to be higher than in populations displaying a lesser degree of phenotypic assortment. This being the case, it would then likely contribute to increased group encounter rates, and result in greater levels of competition for resources, for example, food. Identifying selection pressures that shape decision-making associated with phenotypic assortment during the formation of fish groups, as well as the underlying mechanisms, are key components towards a better understanding of how these decisions impact individual fitness.

Schooling behaviour in teleosts is made possible via an array of sensory mechanisms used to detect and transfer information required for the maintenance of cohesive behaviour in support of group living (Cahn, 1972; Shaw, 1978). Therefore, having identified specific behavioural traits associated with schooling in a New Zealand species (yellow-eyed mullet), we further investigated two key sensory mechanisms underlying those behaviours, namely lateralisation

of visual function, and the lateral line system. This thesis was the first to quantify these sensory systems in yellow-eyed mullet. Results from optic lobe morphology in strongly lateralised juvenile and adult yellow-eyed mullet suggested the possibility of ontogenetic plasticity related to visual lateralisation in this species (Chapter 6). Varied strength and direction of lateralisation in juvenile yellow-eyed mullet, but strong visual bias at a population level in adults, further supports this theory. Behaviourally, results showed that being strongly lateralised conferred an advantage as these fish tended to occupy a position of safety within the school (therefore, considered to increase individual fitness), more frequently than non-lateralised fish. The age-related difference in lateralisation strongly suggests that future research should investigate changes in laterality, and schooling behaviour, throughout an individuals' lifetime. Adding further weight to our theory of ontogenetic plasticity, results also suggest a level of adaptive and/or phenotypic plasticity in rotational swimming bias from differences seen between juvenile and adult yellow-eyed mullet. This is possibly related to both genetic and environmental factors and would also be an important area for future research. Understanding the population genetics that drive ontogeny and plasticity of phenotypic traits, such as sensory systems, is important for improving our understanding of how species maintain collective behaviour. However, research also needs to include differences between *individuals* among populations to elucidate the effect of directional selection on phenotypic traits in fish.

In addition to lateralised visual function, we investigated the lateral line system: a sensory organ used for hydrodynamic sensing, that plays a vital role in the maintenance of schooling behaviour (Faucher et al., 2010; Kasumyan, 2003; Larsson, 2009). Given the fundamental importance of this system to schooling, it is surprising that little is known about its structure or function in an abundant schooling species, such as yellow-eyed mullet. Our results in Chapter 7 clearly showed a highly sensitive trunk-based lateral line system consisting of several hundred thousand innervated hair bundles, the loss of which caused disruption to schooling

behaviour, and decreased object detection. Little was known about the neuroethology providing the framework linking this system to schooling behaviour in yellow-eyed mullet; however, it is clear from the results that it contributes significantly to schooling behaviour. Of particular relevance to this species are the daily changes in abiotic factors within estuaries (e.g. turbidity levels), reducing the functionality of sensory mechanisms for schooling during these conditions, for example vision. Without vision, and to maintain schooling behaviour under differing environmental conditions fish must, therefore, possess more than one sensory modality. The fact that this species has invested in such a highly developed and sensitive lateral line system suggests that schooling is of paramount importance to individual fitness. It is not known if sympatric species possess similar hydrodynamic sensing abilities, and this would be an interesting area of research, for example in kahawai and snapper. Given the similar environmental selection pressures faced by sympatric species, one would expect sensory systems, having evolved independently in species, or retained from ancestors, to have similar morphological characteristics. Interspecific differences in behavioural traits and sensory abilities, and their effect on behavioural interactions in fish species, particularly in wild fish populations, is relatively unknown.

The use of feeding stations is gaining international attention as a sustainable method of increasing commercial fisheries production via on-growth and capture of free-ranging fish populations (Bjornsson, 2011; Zion & Barki, 2012). However, little is known of the interspecific interactions and phenotypic composition of mixed-species fish assemblages, or their potential effects on feeding station productivity. Whilst Chapters 3–5 investigated group behavioural traits in three New Zealand species within a controlled setting, Chapter 8 sought to elucidate behavioural traits associated with interspecific interactions whilst foraging at an estuarine-based feeding station and to compare with tank-based studies. Results from sonar and stereo-video observations showed a high degree of size-assortment in schools, mutual foraging

in yellow-eyed mullet and kahawai, and yellow-eyed mullet maintained high temporal association rates to the feeding station. Repeated avian attacks and competitive interactions with teleosts had no long-term effects, even though a trophic-level hierarchy was evident with the much larger apex predator yellow-tail kingfish (*Seriola lalandi*) dominating foraging at the exclusion of all other species.

There appeared to be a strong degree of habituation of fish associated with predation threat (i.e. diminishing response), which negated the risk of fish avoiding the feeding station permanently as a result. This is an important finding because it suggests that predation risk is not a deterrent to the success of this fish aggregation method. Interestingly, the latency period between the start time of an avian predator strike and resumption of feeding was around 4 s; however, yellow-eyed mullet kept much greater distances away from teleost competitors than avian predators. Given the sudden, unexpected and brief nature of avian predator encounters, compared with the length of time a teleost predator is likely present, this is unsurprising, but does suggest a range of predator avoidance behaviours are being employed under differing perceived threat levels. Combined, these results suggest that the interactions and behavioural traits displayed by wild fish populations utilising a feeding station do not detract from the potential of this fish production method.

9.2 CONCLUSIONS

This thesis investigated behavioural and sensory mechanisms guiding formation and maintenance of schooling in three New Zealand estuarine associated species. Estuarine fish populations are exposed to selection pressures associated with environmental conditions, and shared habitat use among multi-species assemblages (Elliott & Hemingway, 2008). The differences in species behavioural traits shown in this thesis strongly suggests that they contribute to the successful co-existence of multi-species within a shared habitat. Further to that, our increased understanding of interspecific interactions highlights the potential of feeding

stations for recruitment and on-growth of multiple species to augment in-shore fisheries. In terms of sensory function, yellow-eyed mullet have a highly developed trunk lateral line system and an apparent level of ontogenetic plasticity in visual lateralisation. Estuarine habitat is subjected to daily changes in biotic and abiotic factors; for example, multi-species interactions, salinity, tidal flow, and turbidity. Vision is a dominant sensory system in schooling behaviour (Pitcher et al., 1976); however, in low visibility (i.e. high turbidity), fish must be able to rely on other sensory systems to maintain schooling behaviour. In particular, this involves use of the mechanosensory system. Juvenile fish are thought to occupy shallower near-shore estuarine habitats, whereas adults prefer deeper water (Paterson & Whitfield, 2000, K.L. Middlemiss personal observation), which gives support to the theory that growth-related differences in visual bias found in yellow-eyed mullet could be related to different environmental conditions contained within these habitats. Globally, Mugilidae commonly inhabit estuarine ecosystems (Whitfield, 2015), and their successful exploitation of this challenging habitat requires a range of physiological adaptations (Major, 1978). Arguably, their highly developed sensory and behavioural repertoire has evolved as a result of these selective pressures. Genetic and environmental factors are important considerations for the advancement of research on the mechanisms that underlie behavioural traits.

Animal behaviour is inextricably linked with both heritability and adaptive responses to environmental selection pressures (Bell, 2008), collectively known as Gene x Environment (GxE) interactions. Although descriptions of striking diversity in animal behaviour are plentiful, little is known about the mechanisms by which behaviours evolve among populations and species. To fully understand behavioural evolution, it is necessary to identify the genetic mechanisms that mediate behavioural change; however, relatively few studies to date have successfully identified causal genes or genomic regions that contribute to behavioural variation in animals (Bell, 2008). Quantitative genetics provides a statistical framework for partitioning

both trait variation and covariation within and between individuals, and can be used to estimate population and individual level behavioural plasticity. This may prove to be a fruitful area for future research efforts to focus on.

Technological constraints have previously hindered the application of a quantitative genetics framework to dissect the architecture of behaviour (Boake et al., 2002), creating a significant bottleneck in our understanding. A new approach is now conceivable in light of improved sequencing technologies (e.g. whole genome and transcriptome approaches such as Genotyping By Sequencing, GBS), which now allows us to collectively investigate the molecular underpinnings of interactions of related phenotypic traits (including behavioural syndromes) under varied environmental contexts (Cossins & Crawford, 2005; Sih et al., 2004). Genetic analysis of behaviour can also reveal associations between behaviour and morphological or neural phenotypes, providing insight into the proximate mechanisms that control behaviour.

With around 30,000 catalogued species, fish represent the highest vertebrate diversity (Eschmeyer et al., 2010). As a consequence, teleosts display a wide range of behaviours, making them an ideal taxon to investigate the genetic underpinnings of behavioural traits (Bell, 2008). It is recommended that future research address the following hypotheses: (1) sensory and behavioural traits have a genetic component, and (2) genetic and environmental factors interact to produce specific sensory and behavioural traits. This hypothesis-based research should address imperative questions in behavioural ecology by asking how genetics and the environment combine to influence phenotypic traits in fish populations. Results will not only be important for increasing general knowledge of the ecology and collective behaviour in wild fish, but also have important implications for cultured fish populations.

In most countries, selective breeding programmes associated with cultured fish mainly focus on growth rate, which can be linked to selection for more aggressive animals (Gjedrem & Baranski, 2010; Huntingford, 2004). Social interactions among individuals can have profound influences on the expression of performance and welfare traits (North et al., 2006). They can reduce growth due to competition for limited resources and result in mortality due to cannibalism (Baras & Jobling, 2002). The stress effects from high stocking density under intensive culture systems, coupled with such behavioural change, may act against the welfare of genetically improved cultured fish. A strong case can be made for selective breeding programmes to include behavioural traits that may lead to reduced aggression, greater uniformity in harvest weight and that are related to fish health and welfare. Given the timeliness of culture production, there is now an unprecedented opportunity to dissect the genetics of fish behaviour with a focus on GxE. This has further implications for improved fisheries management strategies.

Marine eco-systems are subjected to high fishing pressures and around 4.5 million fishing vessels employ some 57 million people annually (FAO, 2016b). Considering oceans cover around 360 million km^2 (or ~71%) of the Earth's surface (Lutgens, 1992), this equates to around one vessel catching fish for every 60 km^2 of ocean. Global marine capture production continues to increase yearly (currently around 82 x 106 tonnes annually, FAO, 2016b). Efforts to address ways to sustainably increase fish production to meet demands have resulted in the development of methods such as stock enhancement of wild populations. This typically involves augmentation of existing fish populations by releasing cultured juvenile fish. However, the effects of such strategies on wild fisheries is not well understood, and neither are the potential consequences of directional selection on behavioural traits in individuals from cultured populations on wild fish populations. It has been shown that genetic differences resulting from selective breeding in cultured fish can include more aggressive behavioural traits

(Conrad et al., 2011; Huntingford, 2004), which may negatively impact both populations. For instance, released cultured fish may outcompete wild fish for food resources, resulting in decreased survival rates in wild populations if resources are scarce. These are important questions to address as the answers will shape our future understanding not only of behavioural traits in fish, but of ethology in general.

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